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Bacterial Lipopolysaccharide Signaling Through Toll-Like Receptor 4 Suppresses Asthma-Like Responses Via Nitric Oxide Synthase 2 Activity¹

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Asthma results from an intrapulmonary allergen-driven Th2 response and is characterized by intermittent airway obstruction, airway hyperreactivity, and airway inflammation. An inverse association between allergic asthma and microbial infections has been observed. Microbial infections could prevent allergic responses by inducing the secretion of the type 1 cytokines, IL-12 and IFN- γ . In this study, we examined whether administration of bacterial LPS, a prototypic bacterial product that activates innate immune cells via the Toll-like receptor 4 (TLR4) could suppress early and late allergic responses in a murine model of asthma. We report that LPS administration suppresses the IgE-mediated and mast cell-dependent passive cutaneous anaphylaxis, pulmonary inflammation, airway eosinophilia, mucus production, and airway hyperactivity. The suppression of asthma-like responses was not due to Th1 shift as it persisted in IL-12^{-/-} or IFN- γ ^{-/-} mice. However, the suppressive effect of LPS was not observed in TLR4- or NO synthase 2-deficient mice. Our findings demonstrate, for the first time, that LPS suppresses Th2 responses in vivo via the TLR4-dependent pathway that triggers NO synthase 2 activity. *The Journal of Immunology*, 2003, 171: 1001–1008.

Asthma is a chronic respiratory disease characterized by airway inflammation, intermittent airway obstruction, excessive mucus production, airway hyperreactivity (AHR),³ and elevated levels of IgE (1). The mechanisms that cause asthma are complex and vary among population groups and individuals (2) but overall appear to result from an intrapulmonary allergen-driven Th2 response characterized by an increased production of type 2 cytokines (IL-4, IL-5, IL-9, and IL-13) secreted by CD4⁺ T lymphocytes (3).

Over the past decades, the incidence of asthma has risen dramatically worldwide, especially, but not exclusively, in developed countries (2). Because of the short time frame, it is likely that environmental rather than genetic factors are involved. One category of environmental factors that shows consistently a negative association with allergic disorders is microbial infections (4, 5).

In the present study, we evaluated whether bacterial LPS, a prototypic cell wall component of Gram-negative bacteria that activates immune cells via the transmembrane Toll-like receptor 4 (TLR4) (6–8), could suppress asthma-like responses. LPS are ubiquitous in the environment and are often present in polluted air

and in organic or household dusts (9). It has been shown that exposure to airborne LPS can either protect against asthma or exacerbate it (reviewed in Ref. 10). The beneficial effects of LPS are thought to be mediated by enhanced secretion of the type 1 cytokines IL-12 and IFN- γ that are known to down-modulate allergic responses (11, 12). Conversely, airborne LPS might adversely affect asthmatics by enhancing an established airway inflammation and airway obstruction (13, 14).

LPS is also a potent stimulator of NO production (15) and a number of studies have demonstrated the involvement of NO in lung physiopathology (16). In humans with normal airways, exhaled NO is derived from constitutive endothelial and neural nitric oxide synthases (NOS3 and NOS1, respectively), whereas the increased levels of NO detected in asthmatics appear to be derived from inducible NOS (or NOS2) expressed by the inflamed airways (17, 18). Whether NO production has a beneficial or deleterious effect in asthma is still controversial. Data from experimental asthma models, using gene inactivation of NOS isoforms, indicate that the induction of airway eosinophilic inflammation appears to be either dependent or independent of NOS2 activity, whereas NOS1, but not NOS2 expression, seems to be required for protection against AHR (19, 20). In addition, conflicting results were also obtained in studies with drug-induced inhibition of NO production in which NOS2 inhibition has been shown to either exacerbate or attenuate allergen-induced airway inflammation and AHR (21, 22).

It is known that TLR4 is a keystone molecule in the recognition of LPS (23, 24) and that LPS administration results in the production of IL-12, IFN- γ , and NO (15, 25). In addition, NOS2-derived NO might positively or negatively regulate cell signaling (26, 27). We were therefore interested in investigating the effect of LPS on asthma-like responses in TLR4-deficient, IL-12^{-/-}, IFN- γ ^{-/-}, or NOS2^{-/-} mouse strains. In this study, we demonstrate for the first time that LPS signaling through TLR4 suppresses Th2 responses in vivo via NO generated by NOS2 and that this suppression is independent of IL-12 or IFN- γ production.

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³ Abbreviations used in this paper: AHR, airway hyperreactivity; TLR4, Toll-like receptor 4; NOS, NO synthase; PCA, passive cutaneous anaphylaxis; i.n., intranasally; BAL, bronchoalveolar; AG, aminoguanidine; PAS, periodic acid-Schiff; WT, wild type.

Materials and Methods

Mice

Wild-type (WT) C57BL/6J, IL-12^{-/-}, IFN- γ ^{-/-}, and NOS2^{-/-} mouse strains on the B6 background were used throughout these studies. In experiments where passive cutaneous anaphylaxis (PCA) was determined, the following mouse strains were used: BALB/c and C3H/HePas (TLR4^{lps-n}) expressing a functional TLR4 (TLR4n) and C3H/HeJ (TLR4^{lps-d}) expressing a nonfunctional TLR4 (TLR4d). In experiments designed to determine AHR, BP2 mice from Center d'Elevage R. Janvier (Le Genest Saint-Isle, France) were used. All animals, except IL-12^{-/-} mice and C3H/HePas, were obtained as breeding units from The Jackson Laboratory (Bar Harbor, ME). The IL-12^{-/-} mice were kindly provided by Dr. J. Magran through Dr. L. V. Rizzo (Instituto de Ciências Biomédicas (ICB), University of São Paulo, São Paulo, Brazil) and C3H/HePas was obtained from the Institut Pasteur (Paris, France). All mouse strains were bred in our own Animal Breeding Unit (Biotério de Camundongos Isogênicos ICB-USP, Brazil) for many generations under standard pathogen-free conditions. Mice were treated according to Animal Welfare guidelines of both Institutes.

Immunization and induction of allergic airway response

Animals were immunized and challenged with OVA as previously described (28). Briefly, mice were immunized with 4 μ g of OVA (grade V)/1.6 mg of aluminum hydroxide gel in 0.4 ml of PBS on days 0 and 7. On day 14, the mice were challenged intranasally (i.n.) with 10 μ g of OVA/50 μ l of saline to induce an allergic airway response. One week later, mice were rechallenged with i.n. OVA. The control group consisted of nonimmunized mice that received two i.n. instillations of OVA. All determinations were performed 24 h after the last OVA challenge.

Bacterial LPS administration

To study the local vs systemic effect of LPS (*Salmonella abortus equi*) on established airway inflammation, LPS (Sigma-Aldrich, St. Louis, MO) at a dose of 20 μ g/animal was delivered concomitantly with a second OVA challenge either i.n. or i.v., respectively. The dose of LPS was selected based on a dose-response curve as the less toxic one that presented suppressive activity on allergic responses. Moreover, the LPS preparation, at this dose, does not activate macrophages obtained from C3H/HeJ mice (6).

Bronchoalveolar lavage (BAL) fluid

Mice were deeply anesthetized by an i.p. injection of 4 mg/g body wt of chloral hydrate (Labsynth, São Paulo, Brazil) and blood samples from the retro-orbital plexus were collected for nitrate levels and serum Ab determinations. The trachea was cannulated and lungs were lavaged twice with 0.5 and 1.0 ml of PBS. Total and differential cell counts of BAL fluid were determined by hemocytometer and cytospin preparation stained with Instant-Prov (Newprov, Pinhais, Brazil).

Cytokines levels in BAL fluid

The levels of cytokines (IL-4, IL-5, IL-13, and IFN- γ) in the BAL fluid were assessed by a sandwich kit ELISA according to the manufacturer's suggestion and as previously described (28). The following pairs of mAbs were used: 11B11 and biotinylated BVD6–24G for IL-4, TRFK5 and biotinylated TRFK4 for IL-5, and R4-6A2 and biotinylated XMG1.2 for IFN- γ , all purchased from BD Pharmingen (San Diego, CA). For IL-13 determinations, the pairs were 38213.11 and biotinylated goat polyclonal anti-IL-13 from R&D Systems (Minneapolis, MN). Values are expressed as picograms per milliliter deduced from standards run in parallel with recombinant cytokines. The limit of detection was 10 pg/ml for IFN- γ , IL-4, and IL-5 and 31 pg/ml for IL-13.

Determination of airway responsiveness

Airway responsiveness was assessed in unrestrained conscious mice placed in a plethysmographic chamber (Buxco Electronics, Sharon, CT), where respiratory parameters were measured before (1–3 min) and after (3–10 min) an aerosol of methacholine (Sigma-Aldrich) delivered for 20 s at 3×10^{-2} M in the aerosolator. The resistance was expressed as enhanced pause and was calculated as previously described (28).

Determination of OVA-specific IgG1 and IgE Abs

OVA-specific IgG1 and IgE Abs were assayed by sandwich ELISA as previously described (28).

Passive cutaneous anaphylaxis (PCA)

The anaphylactic activity of reagenic Abs was evaluated by passive cutaneous anaphylactic reaction in mice as described by Ovary et al. (29).

Previously shaved mice were injected intradermally with 50 μ l of three serial dilutions of serum in each side of the dorsal skin. After 2 h, they were challenged i.v. with 250 μ g of OVA plus 0.25% of Evans blue solution. All determinations were made in triplicate and the PCA titers were expressed as the reciprocal of the highest dilution that gave a lesion of >5 mm in diameter. The detection threshold of the technique was established at 1/5 dilutions.

Aminoguanidine (AG) hemisulfate treatment

To inhibit NO production, mice were given a 2.5% solution of AG (Sigma-Aldrich) in sterilized drinking water for 3 consecutive days before performing PCA.

Nitrite assay

Serum nitrite concentrations were determined by a spectrophotometric method based on the Griess reaction as previously described (30). Before analysis, nitrate was first converted to nitrite by incubation with nitrate reductase and NADPH (Boehringer Mannheim, Mannheim, Germany) for 6 h at 37°C. Values represent sample data against standard curve.

Lung histology and histocytometry

After BAL collection, lungs were perfused via the right ventricle with 10 ml of PBS to remove residual blood, immersed in 10% phosphate-buffered Formalin for 24 h, and then in 70% ethanol until embedded in paraffin. Tissues were sliced and 5- μ m sections were stained with H&E for light microscopy examination or with periodic acid-Schiff (PAS)/hematoxylin for evaluation of mucus-producing cells. An Integrationsplatte I (Zeiss, Oberkochen, Germany) with a reticule grid was inserted into one eyepiece of the microscopy and small and medium bronchi were examined randomly at $\times 250$ (final magnification). The mucus index was defined as the number of PAS⁺ intersection found in 200 intersections of airway epithelium with the reticule grid.

Statistical analysis

ANOVA was used to determine the levels of difference between all groups. Comparisons for all pairs were performed by Tukey-Kramer honest significant difference test. Values for all measurements are expressed as mean $\pm$ SEM, and *p* values for significance were set to 0.05.

Results

LPS administration suppresses allergic airway inflammation through a mechanism independent of IL-12 or IFN- γ

We first investigated the effect of local or systemic LPS administration on airway eosinophilic inflammation in OVA-immunized B6 (WT) mice. Since LPS induces IL-12 and IFN- γ production and since these cytokines are known to protect against airway inflammation (12, 31), we included IL-12^{-/-} or IFN- γ ^{-/-} mouse strains with the B6 background. The animals were OVA immunized and challenged twice with OVA by the i.n. route to induce an established airway inflammation. In all experiments 20 μ g of LPS was given concomitantly with the second OVA challenge. Fig. 1 shows that immunized and OVA-challenged animals presented more than a 10-fold increase in the total number of cells recovered from the BAL of WT, IL-12^{-/-}, and IFN- γ ^{-/-} mice, as compared with their controls. Differential cell counts revealed that >50% of the infiltrating cells were eosinophils (Fig. 1). These results confirm that our protocol induces an intense eosinophilic inflammation (28). Intravenous LPS administration concomitantly with the second OVA challenge completely suppressed airway eosinophilia in the three mouse strains studied (Fig. 1). Intranasal LPS administration also inhibited airway eosinophilia, but in contrast to i.v. LPS, the total cell number in BAL fluid was not reduced (Fig. 1). In fact, intranasal LPS induced an intense airway neutrophilic inflammation, which was higher in IFN- γ ^{-/-} mice (Fig. 1C) than in the other mouse strains. These results document that regardless of the administration route, LPS inhibits airway eosinophilia through a mechanism that is independent of IL-12 or IFN- γ production. However, although systemic LPS administration totally blocks airway inflammation, local LPS administration

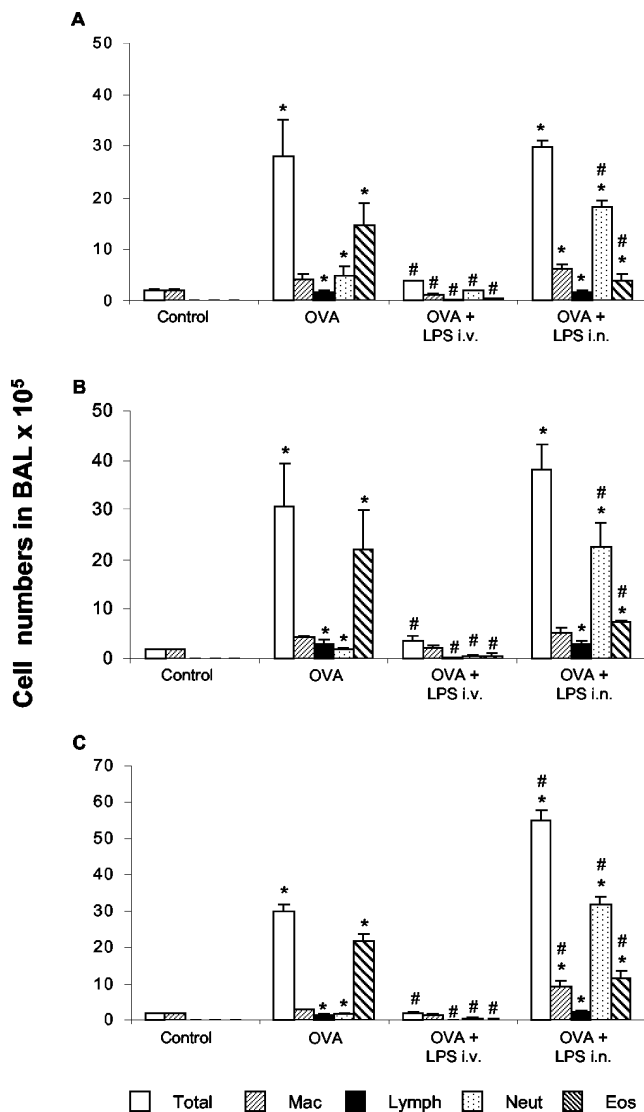


FIGURE 1. LPS suppresses airway allergic inflammation. Groups of WT, IL-12^{-/-}, or IFN-γ^{-/-} mice were OVA-immunized and challenged twice with OVA by the i.n. route. Concomitantly with the second OVA challenge, the animals received 20 μg of LPS i.v. (OVA + LPS i.v.) or i.n. (OVA + LPS i.n.). Control groups consisted of nonimmunized animals that received two OVA challenges. The experiments were performed 24 h after the last OVA challenge and the total and differential cell counts were determined in WT (A), IL-12^{-/-} (B), or IFN-γ^{-/-} (C) mice. Results are expressed as means ± SEM for groups of five mice and are representative of two experiments. *, Significant difference ($p < 0.05$) when compared with the control group. #, Significant difference ($p < 0.05$) when compared with the OVA group.

induces a strong airway inflammation with predominance of neutrophils.

LPS administration suppresses airway type 2 cytokine production through a mechanism independent of IL-12 or IFN-γ

We thereafter determined the cytokine content in the BAL fluid after OVA challenge. Fig. 2 shows that all OVA-immunized mice presented a significant increase of IL-5 and IL-13, but not of IL-4 and IFN-γ, as compared with nonimmunized control animals. The highest levels of IL-5 and IL-13 were found, respectively, in IFN-γ^{-/-} (Fig. 2C, left) and WT mice (Fig. 2A, right). Both routes of LPS administration suppressed type 2 cytokine production but did not affect IFN-γ production (Fig. 2, A–C, right). Thus, LPS inhib-

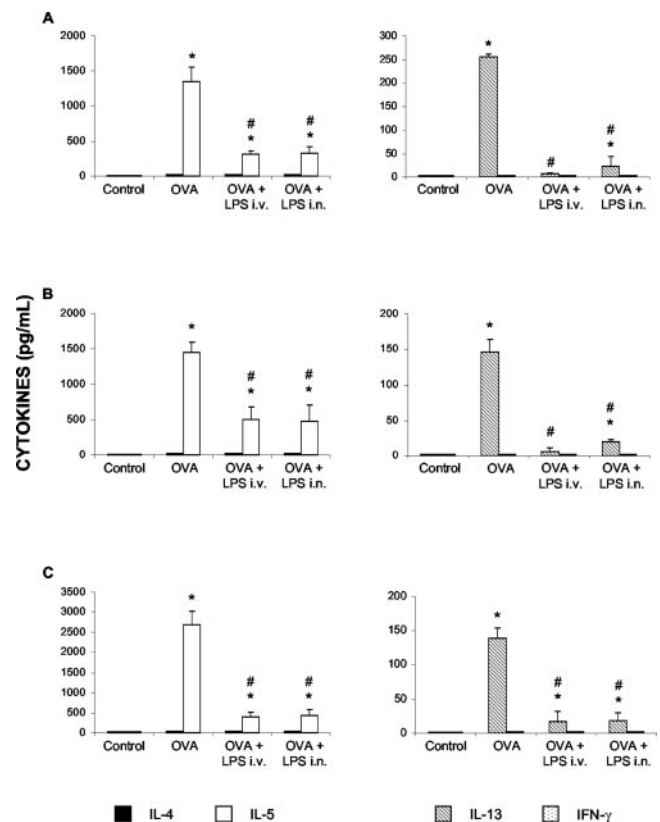


FIGURE 2. LPS suppresses type 2 cytokine production. Groups of WT, IL-12^{-/-}, or IFN-γ^{-/-} mice were OVA-immunized and challenged twice with OVA by the i.n. route. Concomitantly with the second OVA challenge, the animals received 20 μg of LPS i.v. (OVA + LPS i.v.) or i.n. (OVA + LPS i.n.). Control groups consisted of nonimmunized animals that received two OVA challenges. The experiments were performed 24 h after the last OVA challenge and the levels of cytokine in the BAL fluid were determined in WT (A), IL-12^{-/-} (B), or IFN-γ^{-/-} (C) mice. Results are expressed as means ± SEM for groups of five mice and are representative of two experiments. *, Significant difference ($p < 0.05$) when compared with the control group. #, Significant difference ($p < 0.05$) when compared with the OVA group.

its type 2 cytokine (IL-5 and IL-13) production through a mechanism that is independent of IL-12 or IFN-γ.

LPS administration suppresses airway mucus production through a mechanism independent of IL-12 or IFN-γ

It is known that IL-4 and IL-13 are key cytokines involved in mucus production (32, 33). Since we did not detect IL-4 in the BAL fluid and since LPS suppressed IL-13 production, it was of interest to study the mucus production in our experimental model. Fig. 3A shows that immunized mice that received OVA plus LPS, either by the i.v. or by the i.n. route, displayed a significantly lower number of mucus-producing cells as compared with sensitized and OVA-challenged animals. Inhibition of mucus formation by LPS was also observed in IL-12^{-/-} or IFN-γ^{-/-} animals (Fig. 3, B and C).

Systemic but not local LPS administration suppresses AHR

These results above indicate that LPS administration inhibits key features of the OVA-specific Th2-dominated immune response. However, the fact that systemic LPS inhibited airway inflammation, whereas local LPS switched the airway inflammation from eosinophilic to a neutrophilic, led us to investigate the effect of LPS given by these two routes on the development of AHR. In our

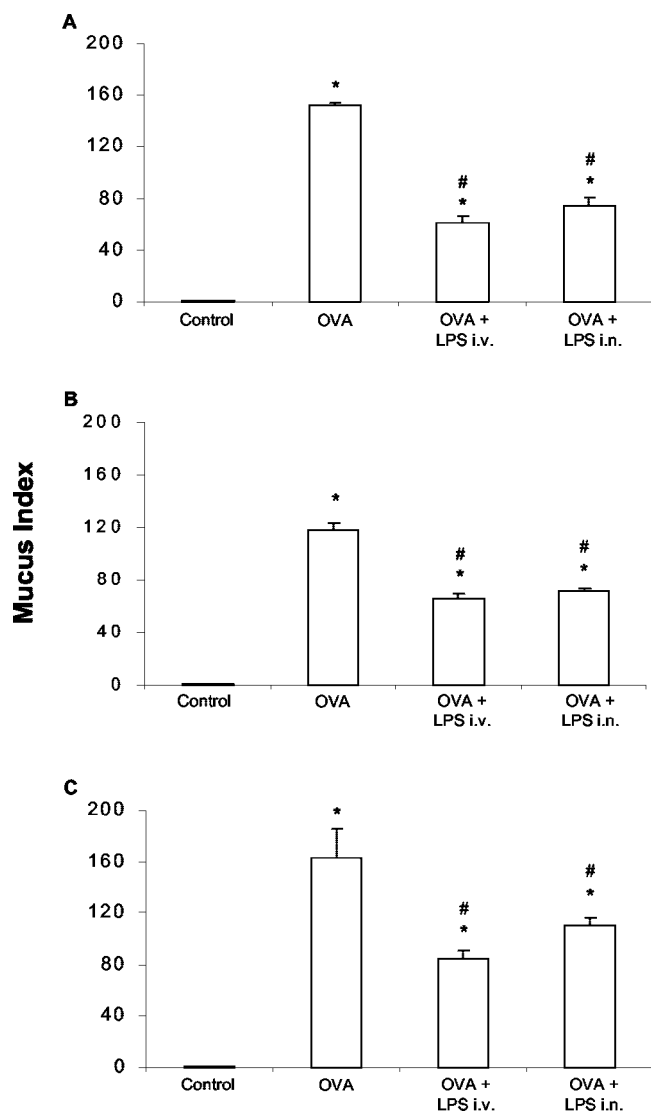


FIGURE 3. LPS suppresses mucus formation. Groups of WT, IL-12^{-/-}, or IFN-γ^{-/-} mice were OVA-immunized and challenged twice with OVA by the i.n. route. Concomitantly with the second OVA challenge, the animals received 20 μg of LPS i.v. (OVA + LPS i.v.) or i.n. (OVA + LPS i.n.). Control groups consisted of nonimmunized animals that received two OVA challenges. The experiments were performed 24 h after the last OVA challenge and the mucus index was determined in WT (A), IL-12^{-/-} (B), or IFN-γ^{-/-} (C) mice. The mucus index (C) was determined in small or medium bronchi by histocytometry by counting 200 PAS⁺ or PAS⁻ intersections of airway epithelium scored with the aid of reticulate grid eyepiece at ×250 magnification. Results are expressed as means ± SEM for groups of five mice and are representative of two experiments. *, Significant difference ($p < 0.05$) when compared with the control group. #, Significant difference ($p < 0.05$) when compared with the OVA group.

model, we found that immunized and OVA-challenged WT animals failed to develop a significant AHR to the spasmogen β-methacholine. Thus, to study AHR we used the BP2 mouse strain, which exhibits consistently larger AHR than most other mouse strains (28, 34). As expected, immunized and OVA-challenged BP2 animals displayed an intense AHR response to β-methacholine (Fig. 4), which was almost abolished in mice after i.v. but not i.n. LPS injection (Fig. 4). Interestingly, animals that were instilled with LPS presented a more prolonged AHR response than OVA-immunized and -challenged animals (Fig. 4).

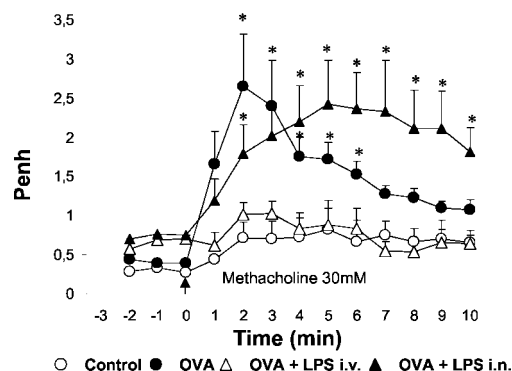


FIGURE 4. Systemic but not local LPS administration suppresses AHR of BP2 mice to methacholine. Groups of BP2 mice were OVA-immunized and challenged twice with OVA by the i.n. route. Concomitantly with the second OVA challenge, the animals received PBS (●) or 20 μg of LPS i.v. (△) or i.n. (▲). Control groups consisted of nonimmunized animals that received two OVA challenges (○). Twenty-four hours after the last OVA challenge, the AHR to methacholine was determined. Results are expressed as means ± SEM for groups of five mice and are representative of two experiments. *, Significant difference ($p < 0.05$) when compared with the control group.

LPS administration, signaling through TLR4, suppresses PCA

Since IgE-mediated mast cell activation initiates the early asthmatic response, we asked whether LPS could also suppress allergic responses that are mediated by anaphylactic Abs and mast cells. Moreover, we also determined whether LPS acts through TLR4 signaling by using TLR4-deficient C3H/HeJ mice (8). Thus, BALB/c, C3H/HePas (TLR4n), or C3H/HeJ (TLR4d) recipients received LPS or PBS 1 h before passive administration of OVA-specific anaphylactic (IgG1 and IgE) Abs. It was found that the PCA titers in BALB/c, TLR4n, and TLR4d recipients were equivalent (~1280) in PBS-treated animals (Fig. 5). In LPS-treated recipients, the PCA titers of BALB/c and TLR4n were suppressed but not in TLR4d recipients (Fig. 5). Thus, LPS blocks early (IgE-mast cell-dependent) allergic response signaling through a TLR4-dependent mechanism.

NOS2^{-/-} mice are resistant to the antiallergic effects of LPS

Since LPS up-regulates NOS2 activity (27) and NO is involved in the suppression of many immunoinflammatory processes (26, 27, 30, 35), we compared the effects of LPS on the Th2-dominated

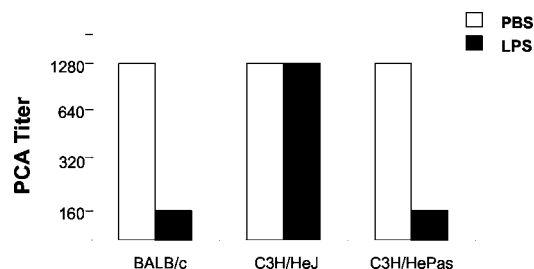


FIGURE 5. LPS suppresses PCA via TLR4. BALB/c, C3H/HeJ (TLR4d), and C3H/HePas (TLR4n) recipients were injected i.v. with PBS or 20 μg of LPS and 1 h later they were injected intradermally with three serial dilutions of serum containing anaphylactic Abs (IgG1 and IgE) in each side of the dorsal skin. Two hours later, they were challenged i.v. with 250 μg of OVA plus 0.25% of Evans blue solution. All determinations were made in triplicate and the PCA titers were expressed as the reciprocal of the highest dilution that gave a lesion of >5 mm in diameter. Five to 10 animals per group were used.

intrapulmonary immune responses of $\text{NOS2}^{-/-}$ with WT mice. Fig. 6A shows that the influx of eosinophils in $\text{NOS2}^{-/-}$ animals after two OVA challenges was similar to that of WT mice. However, after systemic LPS administration, the airway eosinophilia was totally suppressed in WT mice but not in $\text{NOS2}^{-/-}$ mice. Also, LPS administration inhibited mucus formation in WT but not in $\text{NOS2}^{-/-}$ mice (Fig. 6B). LPS also failed to suppress the production of type 2 cytokines in mice lacking NOS2 (data not shown). We then investigated the effect of LPS on PCA in WT,

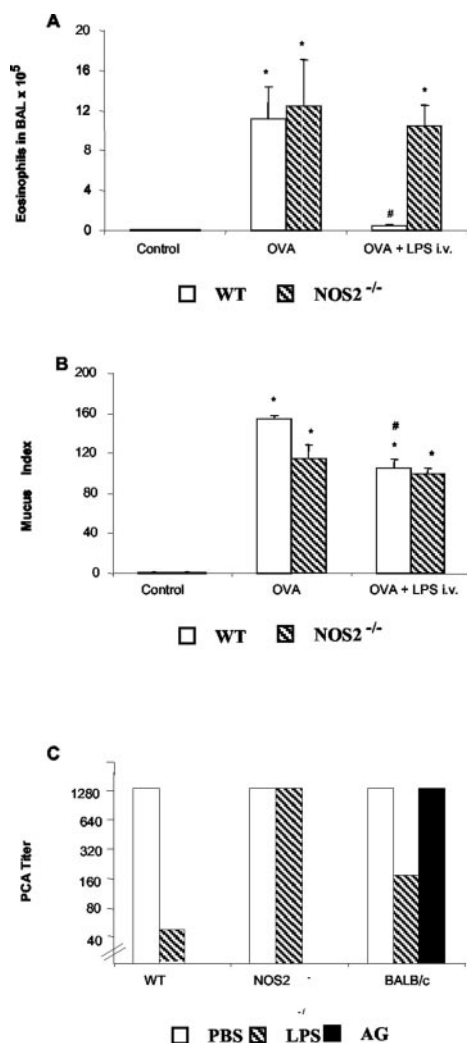


FIGURE 6. $\text{NOS2}^{-/-}$ mice are resistant to the antiallergic effects of LPS. Groups of OVA-immunized WT or $\text{NOS2}^{-/-}$ mice were challenged twice with OVA. Concomitantly with the second OVA challenge, the animals received PBS or 20 μg of LPS i.v. (OVA + LPS i.v.). Control groups consisted of nonimmunized animals that received two OVA challenges. Total eosinophils counts (A) in the BAL fluid and the mucus index (B) were determined 24 h after the last OVA challenge. Results are expressed as means $\pm$ SEM for groups of five mice and are representative of two experiments. *, Significant difference ($p < 0.05$) when compared with the control group. #, Significant difference ($p < 0.05$) when compared with the OVA group. For PCA determinations (C), groups of 10 WT, $\text{NOS2}^{-/-}$, and BALB/c recipients were injected i.v. with PBS or 20 μg of LPS and 1 h later they were injected intradermally with serial dilutions of serum containing anaphylactic Abs. After 2 h, they were challenged i.v. with OVA plus Evans blue solution. For blockage of NOS2 activity, BALB/c recipients ($n = 5$) received water or AG in their drinking water for 3 consecutive days before performing the experiment. The PCA titers were expressed as the reciprocal of the highest dilution that gave a lesion of >5 mm in diameter.

$\text{NOS2}^{-/-}$, and BALB/c recipients. The PCA experiments confirmed the role of NOS2 and NO in mediating the protective effects of LPS, because LPS did not inhibit the PCA reaction in $\text{NOS2}^{-/-}$ animals or in BALB/c recipients that were treated with a NOS2 inhibitor, AG sulfate (Fig. 6C).

LPS suppresses lung pathology via NOS2 activity

Finally, we studied the effect of LPS on lung pathology as assessed by histology and histocytometry in WT and in $\text{IL-12}^{-/-}$, $\text{IFN-}\gamma^{-/-}$, or $\text{NOS2}^{-/-}$ animals. Histology showed that all OVA-immunized mouse strains developed a peribronchovascular inflammation after OVA challenge (Fig. 7, A, C, E, and G). Histocytometric counts revealed that the area (data not shown) and the total number of cells of peribronchovascular infiltrates were higher in $\text{IL-12}^{-/-}$ (7.5 ± 0.07 cells/ μm^2) and $\text{IFN-}\gamma^{-/-}$ mice (7.2 ± 0.9 cells/ μm^2) than in WT (5.0 ± 0.7 cells/ μm^2) or $\text{NOS2}^{-/-}$ (5.2 ± 0.5 cells/ μm^2) animals. Histocytometric counts of LPS-treated animals were similar to control values (1.0 ± 0.05 cells/ μm^2) while LPS-treated $\text{NOS2}^{-/-}$ animals presented 5.8 ± 0.48 cells/ μm^2 . Accordingly, WT, $\text{IL-12}^{-/-}$, and $\text{IFN-}\gamma^{-/-}$ presented virtually normal lung histology (Fig. 7, B, D, and F) whereas $\text{NOS2}^{-/-}$ animals exhibited an intense airway inflammation (Fig. 5H). We also monitored the production of NO by measuring the nitrate serum levels. The levels of nitrate in sera of immunized and OVA-challenged or control mice were comparable to background levels (data not shown). In LPS-treated animals, the mean concentrations of nitrate were 263.9, 348.8, and 126.3 μM for WT, $\text{IL-12}^{-/-}$, and $\text{IFN-}\gamma^{-/-}$ mice, respectively. As expected, the level of nitrate in $\text{NOS2}^{-/-}$ mice after LPS treatment did not increase above the background levels (20 μM). Thus, suppression of lung pathology by LPS is associated with high levels of nitrate in the serum.

Discussion

The present work provides data in support of a role for NO, induced by bacterial LPS signaling through TLR4, in the suppression of key features of asthma. By administering the LPS either i.v. or i.n. we could discriminate between local and systemic effects of LPS. We found that systemic LPS administration, concomitantly with the OVA challenge, almost completely suppressed airway eosinophilia, mucus formation, airway type 2 cytokine production, and AHR. Likewise, nasal LPS administration also suppressed airway eosinophilia, mucus formation, and Th2 cytokine production. However, in marked contrast to systemic LPS injection, animals exposed to LPS by the nasal route displayed a robust AHR and an intense infiltration of neutrophils in the airways. The AHR was determined in BP2 mice because B6 WT animals did not develop significant AHR using our protocol of immunization and challenge. We have previously shown that eosinophils are required for the development of AHR in BP2 animals (34). Thus, the fact that AHR persisted in animals with suppressed airway eosinophilia and type 2 cytokine production is intriguing. The AHR observed in animals treated nasally with LPS might be attributed to the marked influx of neutrophils. However, nasal administration of LPS in nonsensitized animals also induced neutrophilic inflammation but the animals did not present AHR at 24 h (data not shown). It appears that an allergic environment is required for the development of AHR at 24 h after LPS challenge. This hypothesis is in line with the observation that LPS increases experimental allergy when administered directly into the airways (36, 37). More importantly, clinical evidence shows that the presence of LPS in home dust correlates dose-dependently with the intensity of asthma (13, 14). Indeed, it was recently shown that according to the dose of LPS, Th1, or Th2 responses could be generated. At low levels, inhaled

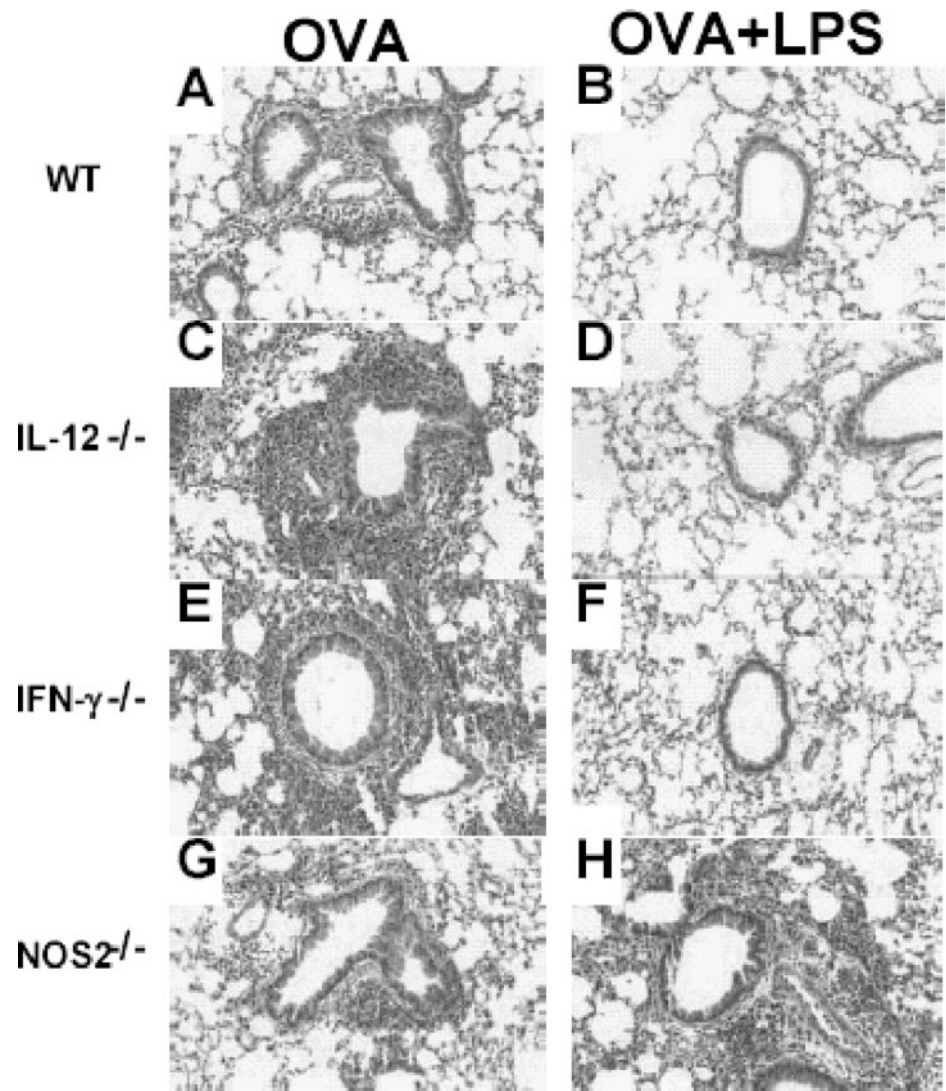


FIGURE 7. LPS suppresses lung pathology via a NOS2-dependent mechanism. Groups ($n = 5$) of WT, IL-12^{-/-}, IFN-γ^{-/-}, or NOS2^{-/-} mice were OVA-immunized and challenged twice with OVA by the i.n. route (OVA). Concomitantly with the second OVA challenge, the animals received 20 μg of LPS i.v. (OVA plus LPS) and 24 h later the lungs were fixed and stained with H&E. Representative lung section showing in OVA groups (A, C, E, and G) peribronchovascular inflammatory cell infiltration and absence of this alteration in OVA plus LPS groups (B, D, and F), except in NOS2^{-/-} mice (H) that presented an intense peribronchovascular inflammation.

LPS signaling through TLR4 induces Th2 responses to inhaled Ags whereas at high levels, inhaled LPS with Ag results in Th1 responses (38). Similarly, nasal LPS administration after allergen challenge completely inhibits AHR and reduces the number of eosinophils and lymphocytes present in BAL fluid while neutrophil influx augments (39). Thus, our results and those described above indicate that the effect of LPS on allergic responses may vary according to dose, timing, and route of administration. It is possible that the detrimental effects of LPS on asthma may be related to the increased severity of the airway inflammation or to increased susceptibility to rhinovirus-induced colds that may result in chronic bronchitis and emphysema with development of irreversible airway obstruction (10).

The beneficial effects of LPS are thought to be due to a re-equilibrium of cytokine pattern. For instance, LPS can induce the production of IL-12 by dendritic cells and provide an important signal for a Th1 shift (40). Alternatively, the lipid A portion of LPS can inhibit IL-4 production by CD4⁺ T cells without inhibiting the production of IFN-γ (41).

In our experimental model, it is unlikely that the inhibition of allergic responses is due to a LPS-induced Th1 shift because 1) we could not detect any increase in the production of IFN-γ in BAL fluid after LPS administration and 2) LPS suppressed the allergic response of animals that were deficient in the production of the two major cytokines (IL-12 and IFN-γ) involved in immune deviation

or suppression of asthma. In line with our findings, it was previously shown that suppression of airway eosinophilia by killed *Mycobacterium vaccae* was not due to a Th1 shift but was dependent on the emergence of allergen-specific regulatory T cells secreting IL-10 and TGF-β (42).

Our results clearly identify NO, derived from NOS2 activity, as the key molecule involved in the suppression of the asthma phenotype. There is increasing evidence for the involvement of NO in airway physiopathology. It is now indisputable that pulmonary NOS2 expression is up-regulated in the lungs of asthmatics (17). However, the role of NOS2 in allergic inflammation is controversial. For instance, Xiong et al. (19) showed that NOS2^{-/-} animals presented an inhibited airway inflammation while De Sanctis et al. (20) found no significant differences in airway inflammation or cellular recruitment into the airway space between NOS2^{-/-} and WT animals. In the same vein, it has been shown that acute inhibition of NOS2 activity either suppresses or exacerbates airway inflammation and chemokine expression (21, 22). Our results and those of others (20) clearly indicate that airway inflammation is fully expressed in the absence of NOS2. Thus, the apparent conflict between studies that favor NO derived from NOS2 as a proinflammatory molecule with those that are opposed to this concept is likely due to differences in the immunization and challenge protocols, and to the concentration of NO produced.

Regarding NOS2 activity, we found that i.v. but not i.n. LPS administration increased serum nitrate levels (data not shown). Also, in our protocol we could not detect any significant increase in the nitrate serum levels of OVA-immunized and -challenged animals. It is possible that NO is generated in the lung after OVA challenge or after i.n. LPS administration at doses that are converted to nitrate and rapidly cleared by the kidneys. In marked contrast, concomitant administration of OVA plus systemic LPS generated high nitrate levels in all mouse strains, except in NOS2^{-/-} animals. The results obtained with the PCA assay, airway inflammation, and lung pathology clearly indicate that LPS signaling via TLR4 activates NOS2 that, in turn, mediates the inhibition of the asthma phenotype. Interestingly, although IFN- γ ^{-/-} mice produced 2-fold lower levels of serum nitrate than WT or IL-12^{-/-} mice after LPS treatment, the pulmonary Th2 responses were totally suppressed. This suggests that at least 50% less NO is required for the suppression of strongly polarized Th2 responses.

Several pieces of evidence indicate that the biological activities of NO may vary depending on the NO concentration (low or high doses exert pro- or anti-inflammatory effects, respectively), the cell types, enzymes, and transcription factors involved (26, 27, 30). Our data suggest that high concentrations of NO are affecting different cell types such as T cells, eosinophils, mast cells, and bronchial epithelial cells. Thus, it is likely that very complex regulatory pathways are operating in vivo in our model, which may particularly include: 1) NO might inhibit the functions of key cell types involved in allergy such as mast cells and Th2 lymphocytes. Indeed, NO was identified as the major molecule that inhibits IgE-mediated degranulation of mast cells (43), a finding that is in line with our observation that NOS2 and NO production were required for PCA inhibition. In addition, we showed that suppression of PCA required LPS signaling through TLR4 as LPS failed to suppress PCA in TLR4-deficient C3H/HeJ mice. It was shown that NO blocks the development of Th1 cells but does not have any effect on Th2 cells (44). Our results and those from others do not support this hypothesis. We showed that induction of NOS2 by LPS suppressed the secretion of pulmonary Th2 cytokines while others have shown that inhibition of NO exacerbates airway hyperresponsiveness, eosinophilia, and C-C chemokines (21). 2) NO may inhibit the expression of cell adhesion molecules (VCAM-1) on endothelial cells that are mediated, in part, by inhibition of *kB* cis-acting elements (45), which in turn might block the influx of inflammatory T cells and eosinophils to the lung. 3) NO may trigger intrapulmonary cell death. It was previously shown that LPS induces intrapulmonary cell apoptosis (46). In our model, it appears that airway eosinophils but not mucus-producing cells are undergoing cell death, because administration of LPS 7 days after the first OVA challenge reduced drastically the number of eosinophils but not mucus-producing cells (data not shown). Thus, NO might be critically involved in eosinophil apoptosis. However, our data are in contrast to previously published work indicating that NO acts as a survival factor for eosinophils (47–49). A recent report also showed that LPS administration in OVA-immunized and -challenged rats drastically decreased an established airway eosinophilia but in conflict to our results, this suppression was associated with a decreased activity of NOS2 (50).

In summary, we have shown that systemic LPS administration almost completely suppresses early and late allergic reactions and AHR in a murine model of asthma. This suppression was exclusively dependent on TLR4 and NOS2 activity but was independent of IL-12 or IFN- γ production. Thus, in addition to the classical concept of microbial-driven Th1 shift, our findings add a novel mechanism for the observed inverse correlation between microbial

infections and asthma (5). Moreover, the data presented identify NO as a potentially relevant molecule for the treatment of acute exacerbation of asthma.

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