

Exposure to Endotoxins during Sensitization Prevents Further Endotoxin-Induced Exacerbation of Airway Inflammation in a Mouse Model of Allergic Asthma

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Key Words

Asthma · Allergy · Endotoxin · Inflammation · Immunoglobulin E · Th2/Th1 cytokines · Mouse

Abstract

Background: We have shown previously that lipopolysaccharides (LPS) inhibited airway inflammation in allergen-sensitized and challenged mice when administered during sensitization, while exacerbating the inflammation when given upon challenge. We have here investigated the effect of LPS administered during both sensitization and challenge on airway inflammation, as well as on the profile of the T-helper (Th) response to allergen. **Methods:** Mice were sensitized and challenged with ovalbumin (OVA), in the presence or absence of effective doses of LPS, namely 1 µg during sensitization and 1 ng during challenge. Inflammation was assessed by measuring cell counts and cytokine levels in bronchoalveolar lavage fluid (BALF). The profile of the Th response was determined by quantifying OVA-specific IgE and IgG2a in serum and Th1/Th2 cytokines in the culture medium of splenocytes and in BALF. **Results:** Allergen-induced airway eosinophilia was increased in mice

exposed to LPS during challenge only when compared with controls, whereas it was similarly reduced in animals exposed during sensitization only and during both sensitization and challenge. Mice exposed to LPS during sensitization only or during both sensitization and challenge also displayed a decrease in IgE and an increase in IgG2a, suggesting a switch in the immune response toward the Th1 profile. This was confirmed by quantification of Th1/Th2 cytokines in culture medium of splenocytes and in BALF. **Conclusions:** Our data demonstrate that exposure to endotoxins during sensitization prevents allergen-induced airway inflammation, as well as its exacerbation triggered by further exposure to endotoxins during challenge, while switching the immune response to allergen from a Th2 to a Th1 profile.

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Introduction

Allergic asthma is a chronic inflammatory disease of the airways characterized by infiltration of CD4⁺ T-helper 2 (Th2) cells and eosinophils in the airways associated with elevated allergen-specific immunoglobulin (Ig) E in

serum [1]. Th2 cells play a central role in the pathogenesis of asthma, as they secrete interleukin (IL)-4 that switches B cells towards IgE synthesis, and IL-5 that promotes eosinophil recruitment [2, 3]. Furthermore, the prevention of Th2 cell responses results in inhibition of allergen-induced IgE production and airway eosinophilia [4].

Endotoxins are cell wall components of gram-negative bacteria that are ubiquitous in the environment and exhibit potent immune-stimulatory and pro-inflammatory activities when inhaled [5]. Endotoxins have thus received much attention in the past few years to tentatively explain the increased incidence and severity of asthma observed in developed countries. However, both epidemiological and experimental studies have reported controversial data, with data showing either a protective or an exacerbating effect on disease development and severity [6–19].

By investigating the effect of LPS at increasing doses in a murine model of allergen-sensitization and challenge, we recently demonstrated that the opposing effects of endotoxins in asthma may be explained by both level and timing of exposure [20]. Indeed, we showed that LPS prevented development of airway inflammation induced by allergen in mice when administered at high doses during sensitization, whereas it exacerbated inflammation when given at low doses upon challenge. However, endotoxins are ubiquitous in the environment, and exposure is likely to occur lifelong. Therefore, in the present study, our objective was to investigate the effect of LPS on allergen-induced airway inflammation when administered to mice at effective doses during both sensitization and challenge. Endotoxins are believed to protect from the development of asthma by stimulating Th1 immunity [21]. Therefore, in the present study, we also assessed the profile of the Th immune response to allergen by measuring levels of allergen-specific IgE and IgG2a in serum and release of Th1/Th2 cytokines in the culture medium of allergen-stimulated spleen cells, as well as in the airways.

Material and Methods

Reagents

Chicken egg albumin (OVA, Grade V), aluminium hydroxide, LPS (*Escherichia coli*, serotype 055:B5), bovine serum albumin (BSA), Tween-20 and extravidin-horseradish peroxidase conjugate were purchased from Sigma Chemical (Saint-Quentin Fallavier, France). Biotinylated anti-mouse IgE (clone R35-118) and IgG2a (clone R19-15) antibodies and 3,3',5,5'-tetramethylbenzidine (TMB) were obtained from PharMingen (San Diego, Calif.). He-

macolor was purchased from Merck (Darmstadt, Germany). Ketamine (Imalgene, Merial, Lyon, France) and xylazine (Rompum, Bayer, Puteaux, France) were purchased from Centravet (Velaine-en-Haye, France).

Animals

BALB/c mice were purchased from Charles River Laboratories (Saint-Germain-sur-l'Arbresle, France) and used at the age of 9 weeks. The animals were maintained under controlled environmental conditions with a 12 h/12 h light/dark cycle. Food (UAR-Alimentation, Villemoisson, France) and tap water were available ad libitum. Animal care, handling and experimentation complied with the EU guide for use of laboratory animals and current French legislation.

Allergen Sensitization and Challenge

Mice were sensitized and challenged with OVA, as previously described [20]. Absence of significant LPS contamination of the OVA was ascertained using the *Limulus* amoebocyte lysate assay. Animals were sensitized on days 1 and 7 by intraperitoneal injections of 50 µg OVA adsorbed with 2 mg aluminium hydroxide in saline. Unsensitized animals received intraperitoneal injections of aluminium hydroxide only. Mice were challenged on days 18, 19, 20 and 21 by intranasal instillations of 10 µg OVA in saline or of saline alone for unsensitized animals. Intranasal administrations (12.5 µl per nostril) were carried out under anesthesia (50 mg/kg ketamine and 3.33 mg/kg xylazine i.p.). Measurements were performed 18–24 h after the last challenge.

Exposure to LPS

Mice were exposed to effective doses of LPS during allergen sensitization and/or challenge, as previously described [20]. During sensitization, 1 µg LPS in saline was injected intraperitoneally 1 h prior to each administration of OVA. During allergen challenge, 1 ng LPS was administered by intranasal instillation together with OVA. Control animals received saline instead of LPS.

Collection of Fluids

Mice were anaesthetized by intraperitoneal injection of ketamine (150 mg/kg) and xylazine (10 mg/kg). Blood was drawn from the abdominal vein for serum determination of OVA-specific IgE and IgG2a. Sera were collected by centrifugation (3,000 g for 15 min at 4°C) and stored at –20°C until assay. After blood collection, the trachea was cannulated to perform bronchoalveolar lavages. Lungs were lavaged by 10 instillations of 0.5 ml ice-cold saline supplemented with 2.6 mM EDTA (saline-EDTA). Fluids recovered from the two first instillations were centrifuged (3,000 g for 5 min at 4°C), and the resulting supernatant was stored at –20°C until cytokine measurements. Fluids recovered from the eight last instillations were dedicated to cell counts.

Serum Immunoglobulin Assay

Serum levels of OVA-specific IgE and IgG2a were measured by enzyme-linked immunosorbent assay (ELISA). Microtiter plates were coated overnight at 4°C with OVA (2 µg/well in 0.1 M NaHCO₃, pH 8.3) and blocked for 2 h at 37°C with 1% BSA in phosphate buffered saline (PBS-BSA). Serum samples diluted in PBS-BSA or a pooled serum from OVA-sensitized animals (internal standard) were then incubated, followed by a biotinylated anti-mouse IgE or IgG2a antibody and an extravidin-horseradish per-

oxidase conjugate. The horseradish peroxidase substrate, TMB was added, and coloration was allowed to develop for 15–20 min. The reaction was stopped by addition of 0.5 M H₂SO₄ and absorbance intensity was read at 450 nm. Sample levels of OVA-specific IgE or IgG2a were expressed as arbitrary units (AU) related to the absorbance of the internal standard.

Total and Differential Cell Counts in BALF

BALF was centrifuged (160 g for 5 min at 4°C) to pellet cells, and erythrocytes were lysed by hypotonic shock. Cells were then resuspended in 500 µl ice-cold saline-EDTA and total cell counts were determined using a hemocytometer (Neubauer's chamber, VWR, Strasbourg, France). Differential cell counts were assessed on cytologic preparations obtained by cytocentrifugation (Cytospin 3, Shandon Ltd., Runcorn, Cheshire, UK) of 200 µl of diluted BALF (250,000 cells/ml in ice-cold saline-EDTA). Slides were stained with Hemacolor and determinations were performed by counting at least 400 cells for each preparation. Differentiated cells were expressed as absolute numbers from total cell counts.

Splenocyte Cultures

Spleens were mashed in 5 ml RPMI-1640 (Gibco, Cergy Pontoise, France) and cells were filtered through a 70-µm cell strainer. After washing in RPMI-1640, red blood cells were lysed and cells were resuspended in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum (Gibco), 1 mM L-glutamine (Gibco), 50 µM β-mercaptoethanol (Sigma), 100 U/ml penicillin (Gibco), 100 µg/ml streptomycin (Gibco) and 2.5 µg/ml gentamicin (Gibco). Cells were then cultured in 96-well plates (4 × 10⁵ cells/well, 37°C, 95% O₂-5% CO₂) with or without OVA (100 µg/ml for 4 days).

Cytokine Assays

Cytokines were quantified in BALF or in supernatants of splenocyte culture by capture ELISA kits according to instructions provided by the manufacturer (PharMingen).

Statistical Analysis

Data are presented as means ± SEM. Statistical differences were analyzed from raw data by the Kruskal-Wallis test followed by the Mann-Whitney U test. Data were considered significantly different when $p < 0.05$.

Results

Effect of Exposure to LPS on Allergen-Induced Airway Inflammation

BALF from unsensitized mice contained few cells (differentiated exclusively as macrophages, not shown) and no neutrophils or eosinophils (fig. 1). OVA-sensitized and challenged mice exposed to saline exhibited a significant increase in total cell number (7.5-fold, $p < 0.001$), when compared with unsensitized mice (fig. 1). This increase was associated with marked ($p < 0.001$) eosinophilia (fig. 1), but no significant neutrophil infiltrate (not shown). Airway eosinophilia induced by allergen sensitization

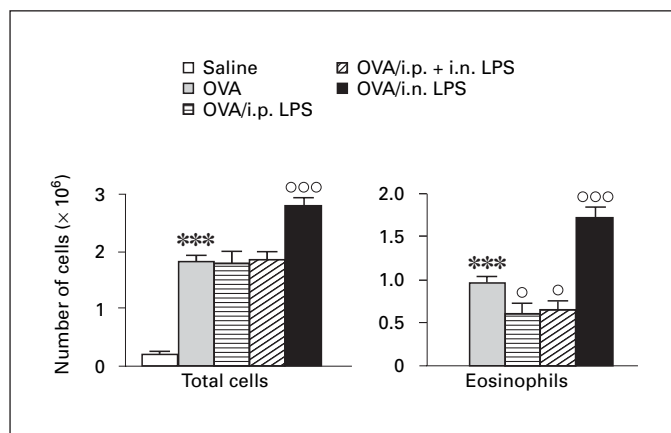


Fig. 1. Effect of exposure to LPS on allergen-induced airway inflammation. Total cell and eosinophil counts in BALF from unsensitized mice (Saline) and from OVA-sensitized and challenged mice exposed to saline (OVA) or to LPS during sensitization (OVA/i.p. LPS), challenge (OVA/i.n. LPS), or sensitization and challenge (OVA/i.p. + i.n. LPS). Data are means ± SEM of 5–11 animals. Statistically significant differences at $p < 0.001$ (***) when compared with unsensitized mice (Saline), and at $p < 0.05$ (°) and $p < 0.001$ (°°°) when compared with OVA-sensitized and challenged mice exposed to saline (OVA).

and challenge was increased by 1.8-fold ($p < 0.001$) in mice exposed to LPS during allergen challenge only, whereas it was reduced by 36 and 31%, respectively ($p < 0.05$ for both) in animals exposed during sensitization only or during both sensitization and challenge (fig. 1). No significant neutrophil infiltrate was observed whatever the LPS treatment (not shown).

Mice sensitized and challenged with OVA and exposed to saline exhibited an increase in the pro-inflammatory cytokine, TNF-α ($p < 0.05$) associated with a decrease in the anti-inflammatory cytokine, IL-10 ($p < 0.05$) in BALF, when compared with unsensitized mice (fig. 2A). TNF-α levels were significantly increased (1.3-fold, $p < 0.05$) in BALF from mice exposed to LPS during challenge only, but not significantly modified in BALF from animals exposed during sensitization only or during both sensitization and challenge (fig. 2A). By contrast, IL-10 levels remained unchanged whatever the LPS exposure.

Effect of Exposure to LPS on Allergen-Induced Ig Production

Serum levels of OVA-specific IgE and IgG2a were significantly increased ($p < 0.001$) in allergen-sensitized and challenged mice exposed to saline, when compared with unsensitized animals (fig. 3). These levels were not mod-

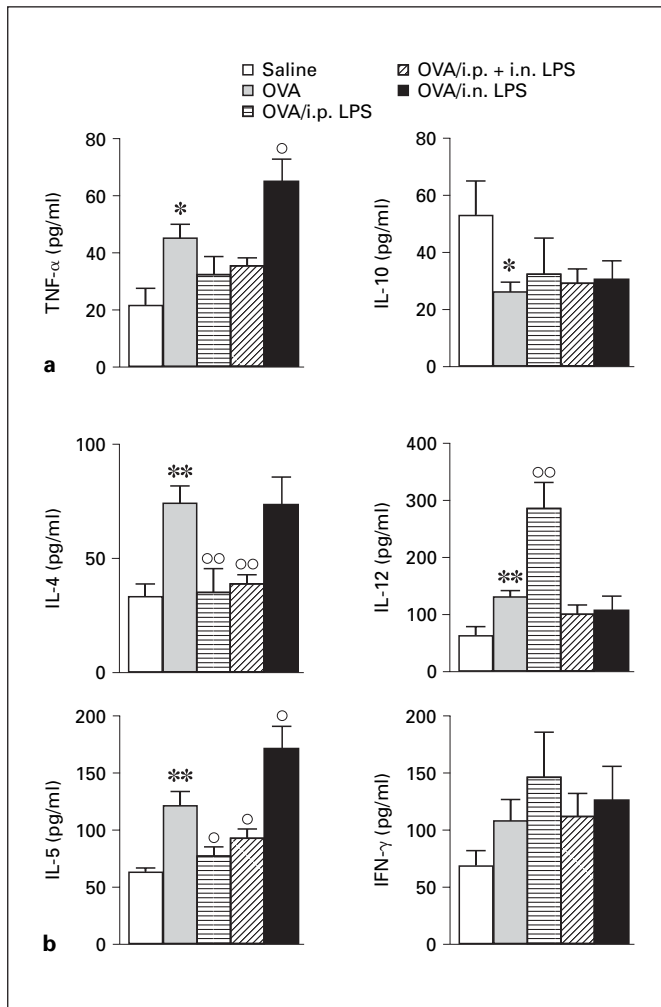


Fig. 2. Effect of exposure to LPS on allergen-induced cytokine levels in BALF. Pro- and anti-inflammatory (a) and Th2/Th1 (b) cytokines in BALF from unsensitized mice (Saline) and from OVA-sensitized and challenged mice exposed to saline (OVA) or to LPS during sensitization (OVA/i.p. LPS), challenge (OVA/i.n. LPS), or sensitization and challenge (OVA/i.p. ± i.n. LPS). Data are means ± SEM of 5–11 animals. Statistically significant differences at $p < 0.05$ (*) and $p < 0.01$ (**) when compared with unsensitized mice (Saline), and at $p < 0.05$ (°) and $p < 0.01$ (°°) when compared with OVA-sensitized and challenged mice exposed to saline (OVA).

ified in OVA-sensitized and challenged mice exposed to LPS during challenge only. By contrast, allergen-sensitized and challenged mice exposed during sensitization only or during both sensitization and challenge displayed a similar decrease in OVA-specific IgE (45 and 47%, respectively, $p < 0.01$ for both) that was associated with an increase in OVA-specific IgG2a [3.5-fold ($p < 0.05$) and 4.2-fold ($p < 0.01$), respectively] (fig. 3).

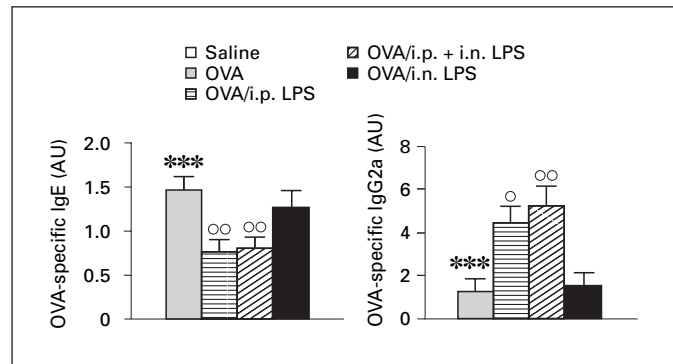


Fig. 3. Effect of exposure to LPS on allergen-induced immunoglobulin production. OVA-specific IgE and IgG2a were measured in serum from unsensitized mice (Saline) and from OVA-sensitized and challenged mice exposed to saline (OVA) or to LPS during sensitization (OVA/i.p. LPS), challenge (OVA/i.n. LPS), or sensitization and challenge (OVA/i.p. ± i.n. LPS). Data are means ± SEM of 5–11 animals. Statistically significant differences at $p < 0.001$ (***) when compared with unsensitized mice (Saline), and at $p < 0.05$ (°) and $p < 0.01$ (°°) when compared with OVA-sensitized and challenged mice exposed to saline (OVA).

Effect of Exposure to LPS on Th2/Th1 Cytokine Production

Th2/Th1 Cytokine Production by Spleen Cells. Spleen cells from unsensitized mice cultured in the presence of OVA produced no or very little of the Th2 cytokines, IL-4 and IL-5, and of the Th1 cytokines, IL-12 and IFN-γ (fig. 4). Splenocytes from OVA-sensitized and challenged mice exposed to saline exhibited a marked increase in IL-4 and IL-5 release ($p < 0.05$ for both), with no significant changes in IL-12 and IFN-γ production when compared with cells from unsensitized mice (fig. 4). The production of both Th2 and Th1 cytokines was not modified in spleen cells from OVA-sensitized and challenged mice exposed to LPS during allergen challenge, when compared with cells from OVA-sensitized and challenged mice exposed to saline. By contrast, the production of IL-4 and IL-5 was dramatically reduced in spleen cells from mice exposed to LPS during sensitization only or during both sensitization and challenge ($p < 0.05$ for all). This decrease in Th2 cytokine release was associated with an increase in the production of Th1 cytokines, IL-12 ($p < 0.05$ for both LPS exposure) and IFN-γ ($p < 0.05$ for LPS exposure during sensitization) (fig. 4).

Th2/Th1 Cytokine Levels in BALF. Mice sensitized and challenged with OVA and exposed to saline displayed a significant increase in IL-4, IL-5 and IL-12 ($p < 0.01$ for all) in BALF when compared with unsensitized animals,

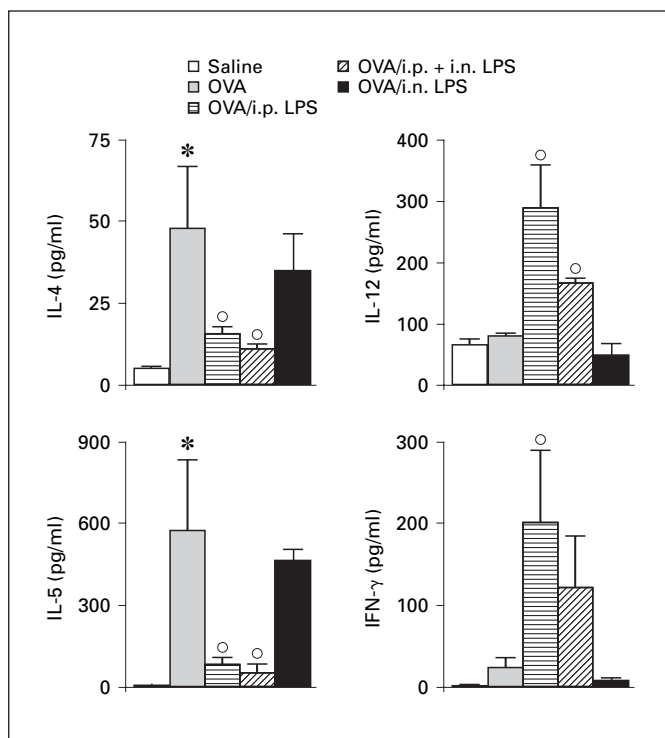


Fig. 4. Production of Th2/Th1 cytokines by spleen cells from unsensitized mice (Saline) and from OVA-sensitized and challenged mice exposed to saline (OVA) or to LPS during sensitization (OVA/i.p. LPS), challenge (OVA/i.n. LPS), or sensitization and challenge (OVA/i.p. ± i.n. LPS) upon *in vitro* stimulation with OVA. Data are means ± SEM of *n* = 3 cultures prepared from 2 animals each. Statistically significant differences at *p* < 0.05 (*) when compared with unsensitized mice (Saline), and at *p* < 0.05 (°) when compared with OVA-sensitized and challenged mice exposed to saline (OVA). Upon culture with medium alone, levels of IL-4 averaged 4.1 ± 0.6, 15.1 ± 2.4, 5.1 ± 1.5, 6.9 ± 1.8 and 6.5 ± 1.8 pg/ml in supernatant of spleen cells from Saline, OVA, OVA/i.p. LPS, OVA/i.n. LPS, and OVA/i.p. ± i.n. LPS animals, respectively. IL-5, IFN-γ and IL-12 remained undetectable in supernatant of spleen cells cultured with medium alone whatever the treatment was.

with no significant changes in IFN-γ (fig. 2b). Levels of IL-5 increased significantly (1.4-fold, *p* < 0.05) in BALF from mice exposed to LPS during allergen challenge only (fig. 2b). In contrast, levels of IL-4 and IL-5 were reduced by 51 and 47% (*p* < 0.01) and by 36 and 24% (*p* < 0.05) in BALF from mice exposed to LPS during sensitization only or during both sensitization and challenge, respectively. In mice exposed to LPS during sensitization only, this was associated with a 2.2-fold increase in IL-12 (*p* < 0.01). However, IFN-γ levels remained unchanged whatever the LPS exposure.

Discussion

In this study, we investigated the effect of effective doses of LPS given during both sensitization and challenge on allergen-induced airway inflammation and on profile of the Th immune response to allergen in the mouse. Our results showed that exposure to LPS during both periods resulted in decreased airway inflammation associated with a switch in the profile of the Th response induced by allergen.

We first showed that LPS administered at an effective dose during allergen challenge failed to exacerbate airway inflammation when given to mice exposed to LPS during sensitization. Indeed, whereas allergen-induced airway eosinophilia was increased in mice exposed to LPS during challenge only when compared with saline-exposed animals, it was reduced in mice exposed during both sensitization and challenge. Likewise, whereas exposure to LPS during allergen challenge only resulted in increased BALF levels of the eosinophil chemoattractant, IL-5, as well as of TNF-α, which enhances eosinophil survival, induces expression of eosinophil chemoattractants, and mediates eosinophil recruitment in allergen-sensitized and challenged mice [22–25], the levels of the two cytokines were decreased in mice exposed to LPS during both sensitization and challenge. Furthermore, mice exposed to LPS during both sensitization and challenge displayed reduced levels of allergen-specific IgE and decreased production of the Th2 cytokines, IL-4 and IL-5, as measured in culture supernatant of spleen cells and in BALF. Exposure to endotoxins during childhood has been largely associated with a lower prevalence of atopic sensitization, hay fever and asthma [7–10]. In keeping with these epidemiological observations, several studies have reported that exposure to LPS during sensitization prevented development of allergen-induced IgE production, Th2 immune response and airway eosinophilia in rodents [12, 14–16, 20]. Interestingly, a recent study reported that the prevalence of atopy, wheeze and asthma is lower in farmers raised on a farm than in farmers with no farm childhood, suggesting that the protective effect of environmental factors, such as endotoxins would continue into adulthood despite ongoing exposure [26]. In agreement with this hypothesis, our data provide experimental evidence that exposure to endotoxins during sensitization may protect towards later exposure to endotoxins at doses that may exacerbate asthma severity.

Inflammation in asthma is under the control of Th2 cells, whereas triggering a Th1 immune response inhibits allergy and asthma [4, 27–30]. Endotoxins are believed

to protect from the development of asthma by stimulating Th1 immunity [21]. However, data provided by experimental studies in this respect remain controversial. Indeed, whereas exposure to LPS during sensitization inhibited allergen-specific IgE production while inducing IgG in rats [12], it reduced Th2 response without enhancing Th1 immune response in mice [14]. In the present study, we showed that allergen-sensitized and challenged mice exposed to LPS during sensitization displayed a decrease in OVA-specific IgE that was associated with an increase in OVA-specific IgG2a. As well, we found that spleen cells from mice exposed to LPS during sensitization released less Th2 cytokines and more Th1 cytokines than cells from mice exposed to saline. Furthermore, allergen-sensitized and challenged mice exposed to LPS during sensitization displayed decreased levels of IL-4 and IL-5 in the airways, which were associated with a marked increase in IL-12. Taken together, our data clearly demonstrated that exposure to LPS during sensitization prevented development of allergen-specific Th2 response, while inducing a Th1 response.

While exposure to LPS during sensitization switched Th cell response to allergen, exposure during allergen challenge triggered no changes in immune response. Indeed, sensitized mice exposed to LPS during challenge only displayed no changes in Ig levels in serum. As well, spleen cells from mice exposed to LPS during challenge produced similar levels of Th2 and Th1 cytokines than cells from mice exposed to saline. The lack of effect of LPS on the systemic immune response in mice exposed during allergen challenge could be attributed to the fact that when instilled in the airways LPS remains restricted to the airways. Another hypothesis could be that exposure

to LPS occurred after expansion of Th2 clones and generation of IgE-producing B cells. In agreement with this hypothesis, Tulic et al. [12] showed that although rats exposed to aerosolized LPS during sensitization exhibited decreased levels of IgE in serum associated with increased levels of IgG, the switch in Ig response vanished when time left between sensitization and LPS exposure increased. By 10 days after primary sensitization, exposure to LPS did not affect Ig production anymore. In the present study, exposure to LPS during challenge also failed to trigger changes in immune response in mice pre-exposed to LPS. Indeed, mice exposed to LPS during both sensitization and challenge displayed Ig levels and Th2/Th1 cytokine profile that were similar to those observed in mice exposed to LPS during sensitization only. Persistence of a switched immune profile most probably accounts for decreased airway inflammation observed in these animals despite exposure to LPS during challenge at doses that may increase allergic inflammation.

In conclusion, our study clearly demonstrates that endotoxins prevent the development of allergic asthma by switching the immune response to allergen from Th2 to Th1, and that this protection, once provided, is effective towards further exposure to endotoxins at doses that may exacerbate asthma severity.

Acknowledgments

This work was supported by the Institut National de la Santé et de la Recherche Médicale, Université Louis Pasteur and Ministère de l'Aménagement du Territoire et de l'Environnement. Carine Delayre-Orthez is supported by a joint PhD grant from ADEME and Région Alsace.

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