

Exposure to Mycotoxins Increases the Allergic Immune Response in a Murine Asthma Model

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Rationale: Epidemiological studies have shown that indoor molds are associated with increased prevalence and exacerbation of respiratory symptoms and asthma. Mycotoxins, secondary metabolites of molds, may contribute to these effects.

Objectives: To investigate the adjuvant activity of mycotoxins on allergic airway inflammation.

Methods: Balb/c mice were exposed via the airways to gliotoxin and via the intestine to patulin, sensitized with ovalbumin (OVA), and then analyzed in acute and chronic murine asthma models. In addition, the effect of mycotoxin exposure on dendritic cell (DC) function was investigated using murine bone marrow–derived DCs.

Measurements and Main Results: Exposure of mice to both mycotoxins enhanced dose-dependently airway hyperreactivity, eosinophilic lung inflammation, and OVA-specific IgE serum levels compared with mice that received only the antigen. These findings correlated with increased Th2 cytokine levels and decreased IFN- γ production. Long-term mycotoxin exposure exacerbated chronic airway inflammation and airway remodeling. *In vitro* or *in vivo* mycotoxin exposure inhibited IL-12 production in maturing DCs and enhanced airway inflammation after adoptive DC transfer into Balb/c mice. Mycotoxin exposure enhanced OVA-induced lung lipid peroxidation and moderately increased isoprostane levels in naive mice. Treatment of mycotoxin-exposed DCs with the antioxidants N-acetylcysteine or glutathione ethyl ester restored IL-12 secretion and pretreatment of exposed mice with N-acetylcysteine prevented the mycotoxin-induced increase of airway inflammation and AHR.

Conclusions: Our results demonstrate that gliotoxin and patulin increase the allergic immune response in mice by modulating the Th1/Th2 balance via direct effects on IL-12 secretion in DCs and by inducing oxidative stress.

Keywords: air pollution; allergy; dendritic cells; Th1/Th2 cells

Asthma is caused by a Th2 cell–mediated immune response to common environmental allergens and is characterized by airway hyperreactivity (AHR) and chronic airway inflammation. The prevalence of atopic diseases like allergic asthma has increased substantially in the last 2 decades (1). The development of allergic diseases is strongly familial and has a genetic basis. However, environmental factors are believed to contribute significantly to this recent increase in prevalence, especially in developed countries. In this context, indoor exposures are of great importance in relation to asthma because most people in industrialized nations spend a large amount of their time indoors (2). Exposure to indoor allergens, such as from house dust mites, animals, and molds, is considered an important risk factor for asthma (3).

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AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Exposure to indoor molds is associated with increased prevalence and exacerbation of respiratory symptoms and asthma.

What This Study Adds to the field

Our results demonstrate that mycotoxins, secondary metabolites of molds, may be responsible for these effects by interfering with dendritic cell function leading to decreased Th1 differentiation and by inducing oxidative stress in the lung.

Although the role of exposure to indoor molds in the development of sensitization and asthma has remained a subject of controversy, epidemiological evidence suggests that molds found in indoor environments are associated with increased prevalence and exacerbation of respiratory symptoms and asthma (4). However, the underlying mechanisms of the allergy-promoting effect of molds remain unclear. Some studies indicate that mycotoxins, secondary metabolites of molds, are responsible for the observed health effects (5, 6). Mycotoxins usually enter the body via ingestion of contaminated food or inhalation of organic dust containing molds or their spores (7). Indoor exposure to mold is predominantly associated with *Aspergillus* and *Penicillium* species, both producing—depending on growth conditions—several mycotoxins, including gliotoxin and patulin (6, 8). Patulin is commonly found in food, such as apple juice and pomaceous fruits (9), and also in the indoor air (10), whereas exposure to gliotoxin is mainly through inhalation of indoor air (7). In several studies, it has been shown *in vitro* that gliotoxin and patulin may affect immune functions (11, 12), although there is still limited understanding about the adjuvant effects of these mycotoxins on immune responses *in vivo*.

The aim of the present study was to examine the role of mycotoxin exposure in allergy development. Therefore, we investigated the effect of patulin and gliotoxin in a murine model of allergic asthma. Our data show that respiratory exposure to gliotoxin as well as exposure to patulin via the intestine clearly exacerbated the asthma-like phenotype. In addition, we demonstrate that both mycotoxins interfere with maturation-associated IL-12 production in dendritic cells (DCs) and IFN- γ production in T cells. The decreased Th1 differentiation may explain the observed adjuvant effect of mycotoxin exposure in a Th2-driven immune response like allergic asthma.

Some of the results of these studies have been previously reported in the form of an abstract (13).

METHODS

Mice

Female BALB/cByJ mice (6–8 wk of age) were obtained from the Elevage Janvier Laboratory (Le Genest St Isle, France), and DO11.10 mice were obtained from the Jackson Laboratory (Bar Harbor, ME).

All animal experiments were performed according to institutional and state guidelines. The Committee on Animal Welfare of Saxony approved animal protocols used in this study.

Immunization and Exposure to Mycotoxins

To induce acute airway inflammation, Balb/c mice were immunized intraperitoneally with ovalbumin (OVA) (20 µg; Sigma-Aldrich, Steinheim, Germany) adsorbed to 2 mg of an aqueous solution of aluminum hydroxide and magnesium hydroxide (Alum; Perbio Science, Bonn, Germany) on Days 1 and 14 followed by 20 µg OVA in 40 µL normal saline given intranasally on Day 14 and Days 17 through 19. Control mice received Alum intraperitoneally and normal saline intranasally. AHR was measured on Day 20 and mice were killed on Day 21. Mice were exposed to gliotoxin (0.1–100 ng in 0.01% dimethyl sulfoxide; Sigma-Aldrich) intranasally and to patulin (5–20 µg in normal saline; Sigma-Aldrich) by gastric intubation on Days 0, 1, 13, 14, 17, and 18. In mycotoxin solutions, no lipopolysaccharide (LPS) was detectable by the limulus amoebocyte lysate test (Cambrex Bio Science, Walkersville, MD). Control mice received either normal saline or 0.01% dimethyl sulfoxide in normal saline. The antioxidant N-acetylcysteine (NAC, 300 mg/kg intraperitoneally) was given to mice 30 minutes before mycotoxin exposure (14). To analyze the effect of mycotoxin exposure on an ongoing allergic inflammation, sensitized mice (OVA; Hyglos GmbH, Regensburg, Germany, endotoxin < 1 EU/mg on Days 1 and 14) were first challenged with OVA on Days 14 and 17 and then exposed to gliotoxin (intranasally), patulin (orally), and OVA on days 18, 21, and 22. In addition, patulin (100 ng) was given intranasally on Day 22. AHR was measured on Day 23 and mice were killed the following day. To induce a chronic asthma-like phenotype, Balb/c mice were immunized intraperitoneally with OVA on Days 1 and 14 and challenged with OVA intranasally twice per week for 8 weeks. Gliotoxin and patulin were applied 1 hour before OVA challenge. AHR was measured on Day 71 and mice were killed the following day.

Measurement of Airway Responsiveness

Airway responsiveness was assessed by methacholine-induced airflow obstruction from conscious mice placed in a whole-body plethysmograph (emka TECHNOLOGIES, Paris, France) as described previously (15, 16). Lung resistance and dynamic compliance were measured by invasive plethysmography (emka TECHNOLOGIES) in response to inhaled methacholine (Sigma-Aldrich). Therefore, mice were anesthetized (100 mg/kg ketamine and 10 mg/kg xylazine; Bayer, Leverkusen, Germany), intubated, and mechanically ventilated at a tidal volume of 0.2 ml and a frequency of 150 breath/min. Baseline lung resistance, dynamic compliance, and responses to aerosolized saline (0.9% NaCl) were measured first, followed by responses to increasing doses (2.5 to 40 mg/ml) of aerosolized methacholine.

Statistical Analysis

One-way analysis of variance and Bonferroni multiple comparison tests were used to determine the statistical significance of differences between groups (Prism 4.0; GraphPad Software, La Jolla, CA). Data were expressed as mean ± SEM. *P* values of less than 0.05 were considered significant.

RESULTS

Exposure to Mycotoxins Increases the Acute Asthma-like Phenotype in OVA-sensitized Mice

To investigate the role of mycotoxins in the development of the allergic immune response we exposed mice to gliotoxin and patulin during OVA sensitization in our established acute asthma model (15–17). Respiratory exposure to gliotoxin and exposure to patulin via the intestine exacerbated airway inflammation in the lung. This was demonstrated by an increase in inflammatory infiltrates in hematoxylin and eosin-stained lung sections (Figure 1A) and verified by objective, investigator-independent computer analysis (Figure 1B). In addition, periodic acid-Schiff-stained lung sections showed an increase in mucus production in the mycotoxin-exposed mice compared with the OVA control ani-

mals (Figure 1A and Figure E1A in the online supplement). Both mycotoxins enhanced the number of eosinophils in the bronchoalveolar lavage (BAL) fluid in a dose-dependent manner (Figure 1C) compared with mice that received only the antigen. These findings are associated with increased serum levels of OVA-specific IgE (Figure 1D) and IgG₁ (Figure E1B) of sensitized mice exposed to mycotoxins, whereas an effect on IgG_{2a} levels was not observed (data not shown).

Exposure of OVA-immunized mice to gliotoxin or patulin increased methacholine-induced AHR as measured in a whole-body plethysmograph for mice (Figure 2A). This was confirmed by assessing AHR using direct invasive assays for lung resistance and dynamic compliance in intubated mice (Figures 2B and 2C). In parallel, in OVA-sensitized mice exposed to mycotoxins we measured increased Th2 cytokine levels, specifically IL-5 and IL-13, and decreased levels of the Th1 cytokine IFN-γ in cell culture supernatants of OVA-restimulated splenocytes (Figure 2D) and mediastinal lymph node cells (Figure E1C).

In summary, exposure of mice to gliotoxin and patulin during OVA sensitization dose-dependently increased the asthma-like phenotype. These effects were accompanied by a reduced release of the Th1 cytokine IFN-γ and an increase of Th2 cytokine production.

Exposure to Mycotoxins Exacerbates Chronic Airway Inflammation and Airway Remodeling

To test the effect of long-term exposure to mycotoxins on the development of chronic inflammation and airway remodeling, we exposed mice to gliotoxin and patulin in an established chronic asthma model (15, 18). The number of eosinophils and lymphocytes in the BAL fluid and the chronic airway inflammation of mycotoxin-exposed mice were significantly enhanced compared with control mice, whereas OVA-specific IgE serum levels and AHR were slightly but not significantly increased (Figures 3A–3D). Furthermore, exposure to mycotoxins exacerbated several features of airway remodeling. The amount of total lung collagen was slightly but not significantly enhanced, and the subepithelial deposition of extracellular matrix proteins examined by Martius Scarlet Blue-stained lung sections, which has been shown to be specific for collagen and old fibrin (19, 20), was increased in mycotoxin-exposed mice compared with control mice that had been only immunized to OVA (Figures 3E and 3F). To show the effect of long-term exposure to mycotoxins on smooth muscle cells and their proliferation in OVA-sensitized mice, we stained lung sections for α-smooth muscle actin and proliferating cell nuclear antigen. There was a significant increase in the smooth muscle area (α-smooth muscle actin-stained cells) in mice exposed to the mycotoxins and in the area of proliferating smooth muscle cells of patulin-exposed mice, whereas gliotoxin-exposed mice showed slightly but not significantly enhanced proliferating cell nuclear antigen-positive-stained cells (Figures 3G and 3H). Mycotoxin exposure of naive mice showed no effect on airway inflammation, IgE levels, AHR, or cytokine production in the acute or the chronic asthma model and no influence on airway remodeling (data not shown).

Mycotoxins Inhibit IL-12 Production in LPS-Stimulated Murine DCs and Decrease Intracellular Glutathione Levels

The increased asthma phenotype after *in vivo* exposure to gliotoxin or patulin was characterized by a reduced Th1 cytokine pattern and an enhanced Th2-mediated allergic response. For the induction of Th1 immune response, the initial secretion of IL-12 by DCs is important (21). To analyze the effect of mycotoxin exposure on DC phenotype and function, immature murine bone marrow-derived DCs were exposed to gliotoxin and patulin and stimulated with LPS, which is known

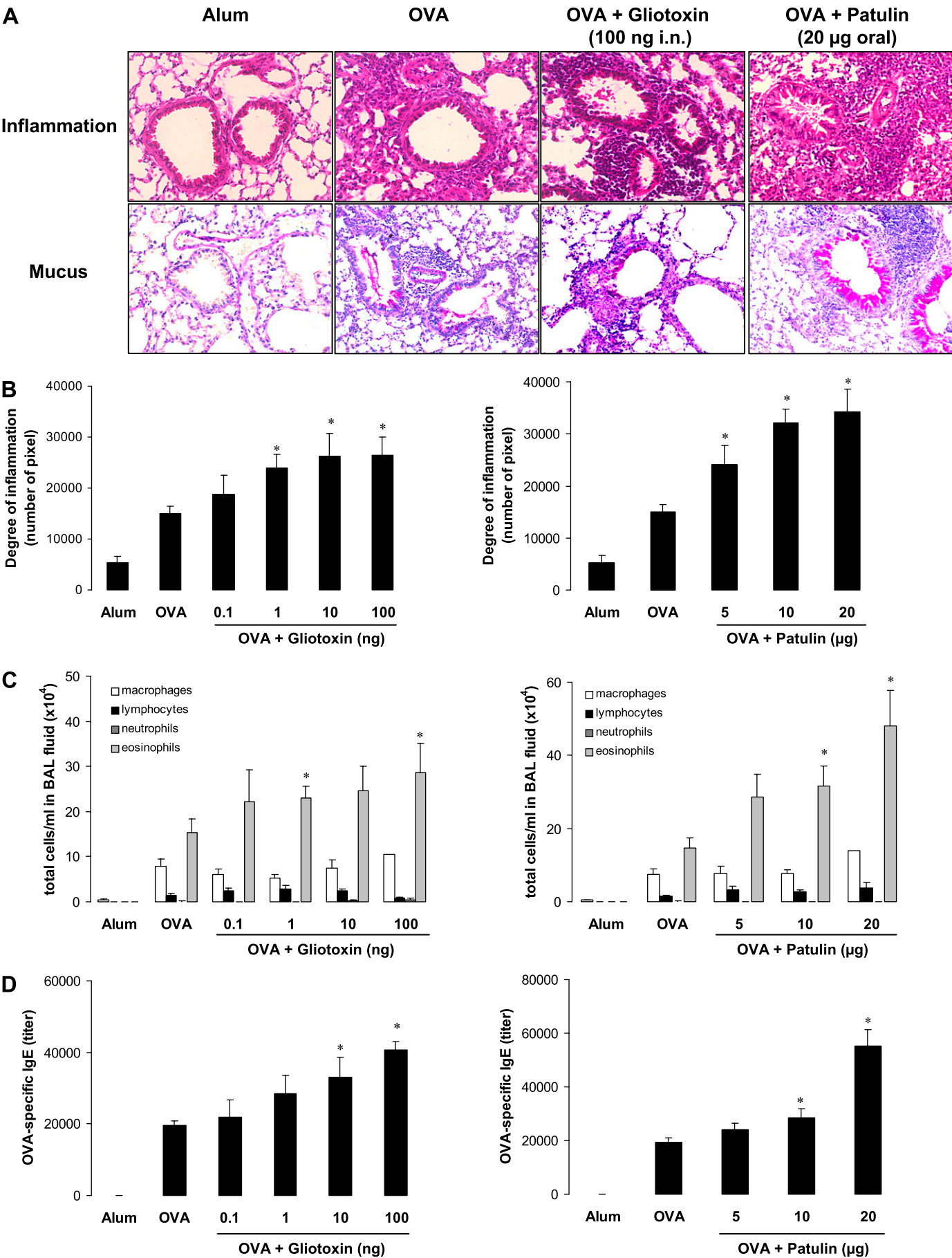


Figure 1. Exposure to mycotoxins increases acute airway inflammation and antigen-specific IgE levels in ovalbumin (OVA)-sensitized Balb/c mice. Balb/c mice were immunized with OVA (Days 1 and 14) and then challenged with OVA on Day 14 and Days 17 through 19. Mice were exposed to mycotoxins and challenged with OVA on Days 0, 1, 13, 14, 17, and 18. (A) Effect of mycotoxin exposure on inflammation (hematoxylin and eosin, $\times 200$) and mucus hypersecretion (periodic acid-Schiff, $\times 200$), (B) verified by an objective, investigator-independent computer-based quantification of lung inflammation. Cell numbers in (C) the bronchoalveolar lavage (BAL) fluid and (D) OVA-specific IgE levels were enhanced dose-dependently after exposure to mycotoxins compared with the unexposed group. Data are expressed as mean $\pm$ SEM, $n \geq 9$ animals per group; $*P < 0.05$ versus OVA, one-way analysis of variance and Bonferroni multiple comparison test.

to induce maturation-associated IL-12 production in DCs (21). Both mycotoxins inhibited the LPS-induced IL-12 production in DCs in a dose-dependent manner (Figures 4A and 4B). IL-10 levels were not affected (data not shown). The concentrations used had no effect on cell viability as measured by trypan blue assay (data not shown). Furthermore, the presence of gliotoxin and patulin caused a reduction in DC maturation, determined by means of CD80, CD86, and major histocompatibility complex II expression (Figure E2).

Previous studies have shown that intracellular glutathione levels play a central role in determining the Th1/Th2 polarization of DCs, whereby decreased glutathione levels correspond to a Th2 response (22). Treatment of LPS-stimulated DCs with gli-

toxin or patulin reduced total glutathione in a concentration-dependent manner (Figures 4C and 4D). Furthermore, pretreatment of mycotoxin-exposed DCs with the thiol antioxidants NAC or glutathione ethyl ester (GSH-Et) partially recovered the LPS-stimulated IL-12 secretion (Figure 4E). NAC and GSH-Et alone had no significant effect on the LPS-induced IL-12 production (data not shown).

Mycotoxin-induced Increase of Th2 Immune Response Is Mediated by Reduced IL-12 Production of DCs

To verify the role of DCs during mycotoxin exposure, OVA-sensitized Balb/c mice were exposed to gliotoxin or patulin and DCs of these mice were isolated from spleen cells using micro beads (see

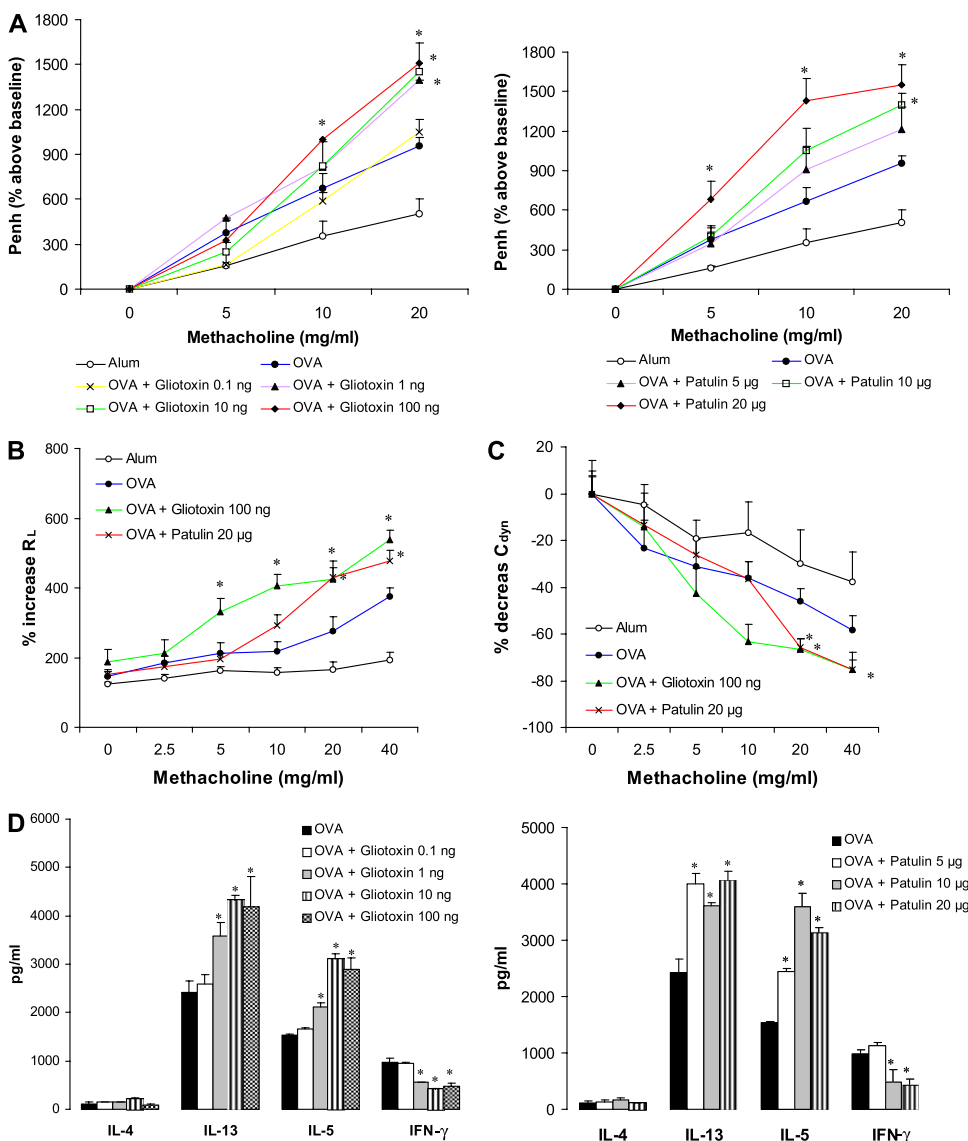


Figure 2. Exposure to mycotoxins increases airway hyperresponsiveness (AHR) and Th2 cytokine production and reduces IFN- γ levels in ovalbumin (OVA)-sensitized Balb/c mice. Balb/c mice were immunized with OVA (Days 1 and 14) and then challenged with OVA on Day 14 and Days 17 through 19. Mice were exposed to mycotoxins and challenged with OVA on Days 0, 1, 13, 14, 17, and 18. Effect of mycotoxin exposure on AHR, determined as (A) enhanced pause (Penh), (B) lung resistance (R_L), (C) dynamic compliance (C_{dyn}), and (D) cytokine levels in the supernatant of OVA-restimulated splenocytes. Data are expressed as mean $\pm$ SEM, $n \geq 9$ animals per group; $*P < 0.05$ versus OVA, one-way analysis of variance and Bonferroni multiple comparison test.

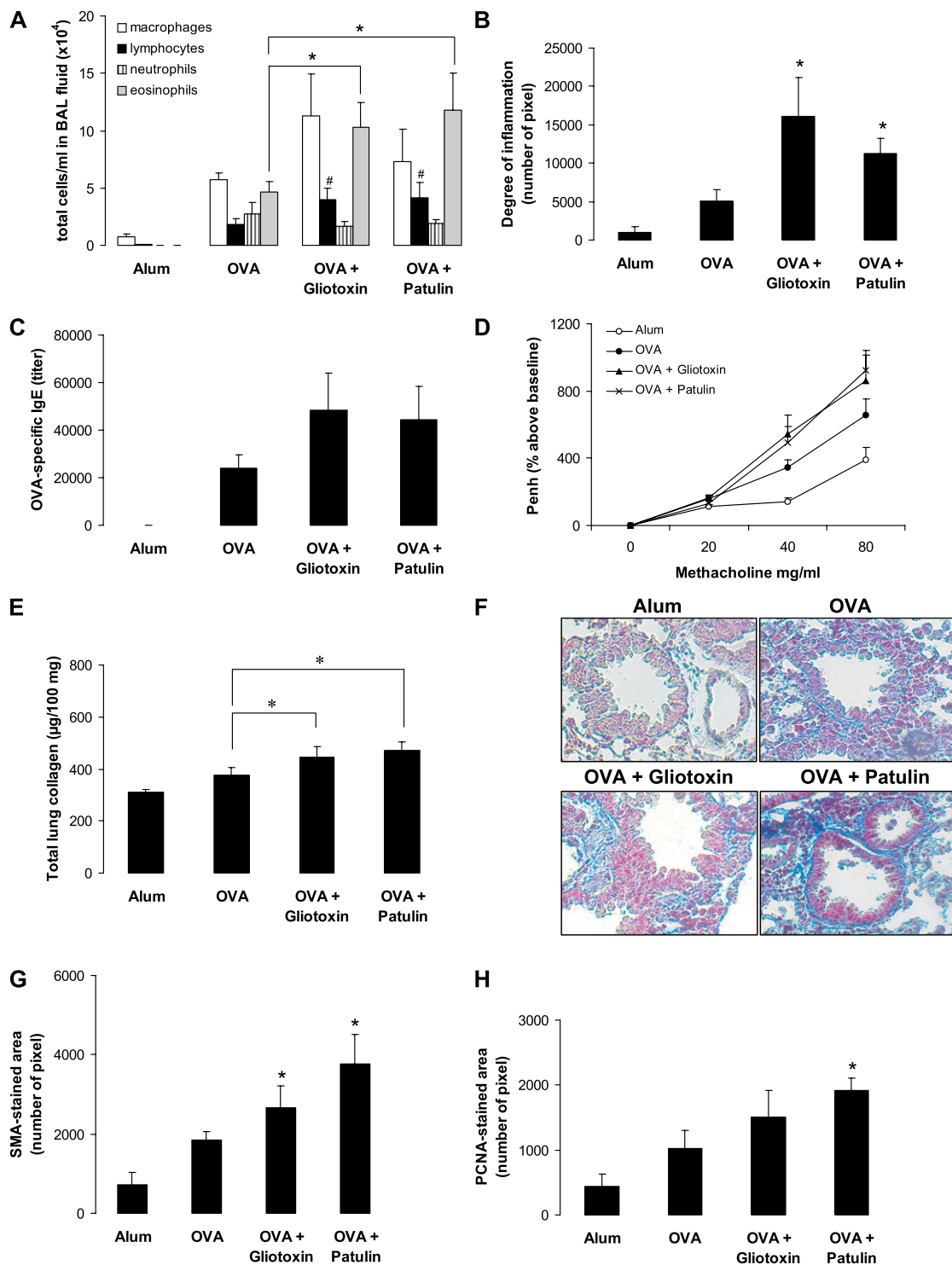


Figure 3. Exposure to mycotoxins exacerbates chronic airway inflammation and airway remodeling. To induce a chronic asthma phenotype mice were immunized with ovalbumin (OVA) (Days 1 and 14) and then challenged with the antigen twice per week for 8 weeks. Effect of exposure to gliotoxin (100 ng intranasally) or patulin (20 μg orally) on total cell numbers in (A) bronchoalveolar lavage (BAL) fluid, (B) inflammation of lung tissues, (C) IgE levels, (D) airway hyperresponsiveness (AHR), (E) total lung collagen, (F) Martius Scarlet Blue-stained lung tissues ($\times 100$), and (G, H) α -smooth muscle actin- and proliferating cell nuclear antigen-stained lung sections. Data are expressed as mean $\pm$ SEM, $n \geq 9$ animals per group; * $P < 0.05$ versus OVA (eosinophils), # $P < 0.05$ versus OVA (lymphocytes), one-way analysis of variance and Bonferroni multiple comparison test. Penh = enhanced pause.

additional METHODS information in the online supplement). The isolated DCs were cocultured *in vitro* with OVA-specific CD4⁺ T cells isolated from OVA-immunized DO11.10 mice, which are transgenic for T-cell receptor recognizing OVA-specific peptide (23). IL-12 production in the coculture with DCs isolated from mycotoxin-exposed mice was significantly reduced compared with the coculture with DCs from unexposed mice (Figure 5A). Moreover, in the cocultures of DO11.10 T cells with DCs isolated from mycotoxin-exposed mice we observed decreased IFN- γ levels, a significant enhancement of IL-5 release for both mycotoxins, and significantly increased levels of IL-13 in the coculture of DCs from patulin-exposed mice (Figure 5B). Interestingly,

exposure of DO11.10 mice to mycotoxins and coculture of isolated CD4⁺ T cells from these mice with CD11⁺ cells from unexposed Balb/c mice also significantly decreased IFN- γ production after restimulation with OVA but had no effect on IL-5 and IL-13 levels (Figure E3).

In an additional experiment we pulsed DCs with OVA and exposed the cells with the mycotoxins for 24 hours. It should be noted that the OVA used for *in vivo* sensitization and for the treatment of DCs was contaminated with LPS, which was confirmed with the limulus amoebocyte lysate test (endotoxin activity: 36.04 ± 2.80 EU/20 μg OVA, which correspond to 3.6 ng LPS). Although in the supernatant of OVA-treated (Sigma) DCs in-

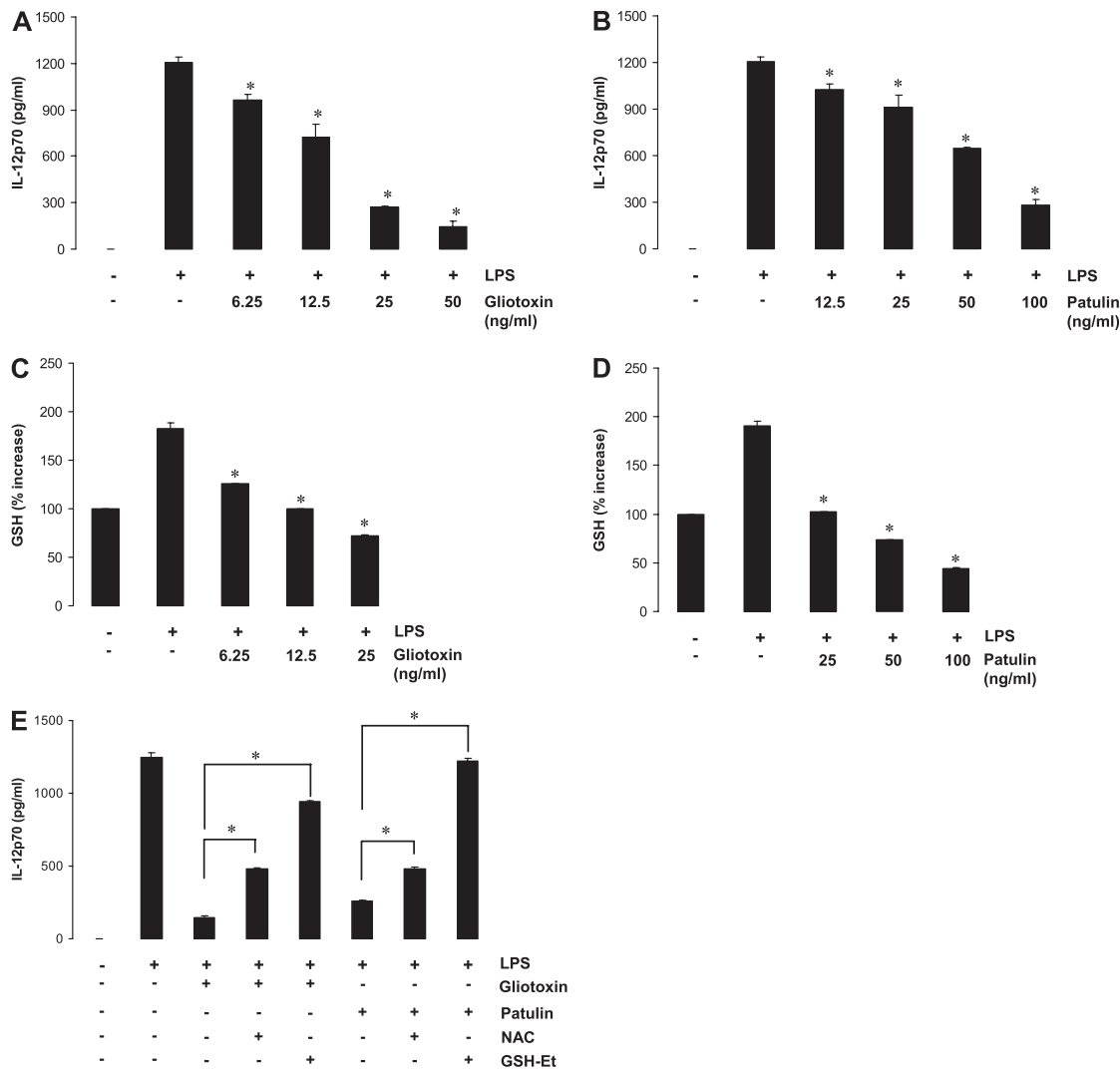


Figure 4. Mycotoxins inhibit IL-12 production in lipopolysaccharide (LPS)-stimulated murine dendritic cells (DCs) and decrease intracellular glutathione levels *in vitro*. (A, B) Effect of exposure of LPS-stimulated bone marrow-derived DCs to gliotoxin and patulin on IL-12 production, and (C, D) intracellular glutathione (GSH) levels. (E) Pretreatment with N-acetylcysteine (NAC) (2 mM) or GSH-ethyl ester (GSH-Et) (0.4 mM) on IL-12 production after gliotoxin (25 ng/ml) and patulin (100 ng/ml) exposure is shown. Data are expressed as mean $\pm$ SEM; $n = 4$ –5/group. * $P < 0.05$ versus LPS, one-way analysis of variance and Bonferroni multiple comparison test.

creased levels of IL-12 were found, the LPS-depleted OVA (EndoGrade OVA) did not induce IL-12 production (Figure E4A). In the following experiment DCs were pulsed with OVA or also exposed to the mycotoxins for 24 hours and then transferred intratracheally into naive Balb/c mice. Mice that received mycotoxin-treated and OVA-pulsed DCs showed an enhanced AHR (Figure 5C), eosinophilic inflammation in the BAL fluid (Figure 5D), airway inflammation in hematoxylin and eosin-stained lung sections quantified by an objective, investigator-independent computer analysis (Figure 5E), and increased levels of Th2 cytokines and reduced IFN- γ production in mediastinal lymph nodes (Figure 5F) and in lung cells (Figure E4B) compared with mice that received only OVA-pulsed DCs.

Reduced DC Function after Mycotoxin Exposure Is Associated with Inhibition of Nuclear Factor- κ B Translocation or p38 Mitogen-activated Protein Kinase Activity

Stimulation of DCs with LPS has been shown to activate nuclear factor- κ B (NF- κ B) as well as p38 mitogen-activated protein kinase (MAPK) pathways, which are involved in transcriptional activation of genes encoding for maturation receptors and IL-12 (24). To determine whether mycotoxins interfere with these pathways we first observed the translocation of NF- κ B in the presence of gliotoxin or patulin. Exposure of DCs to gliotoxin significantly abrogated the LPS-induced NF- κ B translocation,

whereas patulin exposure had no effect (Figures 6A and 6B). On the other hand, patulin significantly inhibited the LPS-induced p38 MAPK activity (Figure 6C), whereas gliotoxin showed no effect under these conditions. Interestingly, the inhibition of NF- κ B translocation by gliotoxin and the reduced p38 MAPK activity after patulin exposure were abolished by pretreatment of the LPS-stimulated DCs with GSH-Et (Figures 6A–6C).

Exposure to Mycotoxins during Antigen Challenge Increases Airway Inflammation, IgE Levels, and AHR, and Induces an Induced Lipid Peroxidation

To investigate whether mycotoxin exposure has an effect on an ongoing allergic inflammation in already sensitized mice we exposed mice to mycotoxins only during antigen challenge. Furthermore, to exclude LPS-mediated effects we used endotoxin-free OVA. Exposure of OVA-sensitized mice to gliotoxin or patulin during the antigen challenge also enhanced the asthma-like phenotype with increased airway inflammation, AHR, and OVA-specific IgE levels (Figures 7A–7D). Exposure of nonsensitized mice to the mycotoxins had no significant effect on these asthma parameters, although the AHR was mildly increased. To find out whether mycotoxins themselves may already induce an inflammatory response in the lung involving cell types other than DCs, we measured several inflammatory cytokines in lung homogenates. We found no significant effects of mycotoxin exposure to unsensitized mice on the thymic stromal lymphopoietin, tumor

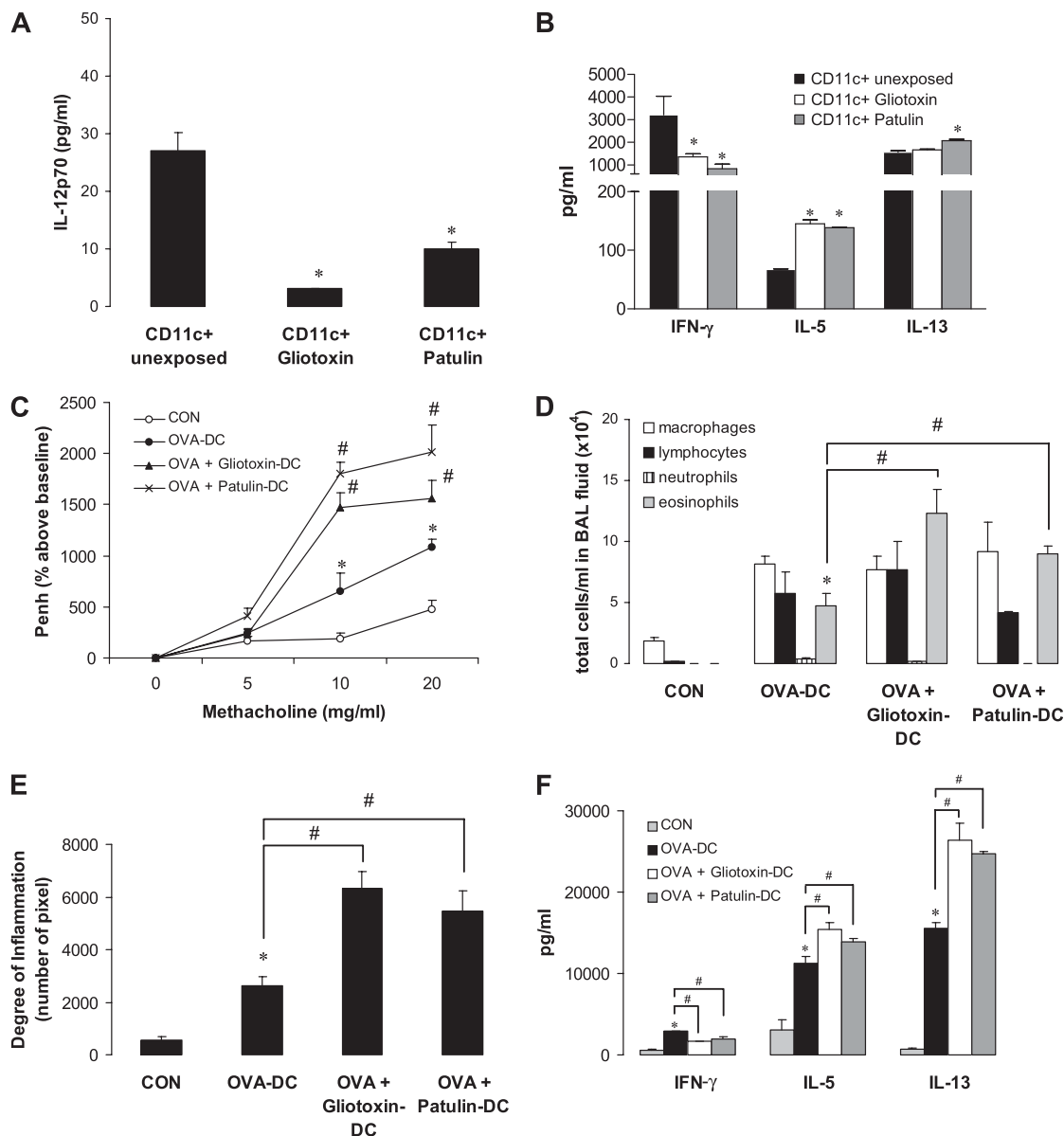


Figure 5. Mycotoxin-induced increase of Th2 immune response is mediated by reduced IL-12 production by dendritic cells (DCs). Splenic DCs isolated from immunized mycotoxin-exposed or unexposed Balb/c mice were cocultured with ovalbumin (OVA)-specific CD4⁺ T cells isolated from DO11.10 mice and stimulated with OVA. (A) IL-12, (B) IFN-γ production, and IL-13 and IL-5 levels are shown. DCs were pulsed for 24 hours with OVA (200 μg/ml), some cells in the presence of gliotoxin (25 ng/ml) or patulin (100 ng/ml), and transferred into the trachea of naive Balb/c mice. As control (CON), DCs only treated with lipopolysaccharide (LPS) (100 ng/ml) were transferred. From Day 10 onward, mice were challenged intranasally with OVA for 3 consecutive days. Effect of DC transfer on (C) airway hyperresponsiveness (AHR), (D) eosinophilic inflammation in the bronchoalveolar lavage (BAL) fluid, (E) airway inflammation in hematoxylin and eosin-stained lung sections quantified by objective, investigator-independent computer

analysis, and (F) cytokine production in mediastinal lymph nodes. Data are expressed as mean ± SEM; n = 4–5/group. *P < 0.05, CD11⁺ unexposed versus CD11⁺ exposed (A, B), CON versus OVA-DC (C–F); #P < 0.05, mycotoxin-DC versus OVA-DC; one-way analysis of variance and Bonferroni multiple comparison test. Penh = enhanced pause.

necrosis factor-α, IL-6, or IL-25 levels in the lung. However, these inflammatory cytokines are increased in antigen-sensitized and -challenged mice, but are not affected by mycotoxin exposure (Figure E5A–D).

To evaluate the role of oxidative stress for the increased allergic inflammation of the lung caused by mycotoxin exposure, 8-isoprostane levels as marker for lipid peroxidation were analyzed in lung homogenates (25). Here, mycotoxin exposure to nonsensitized mice led to an elevated isoprostane concentration that was significant for gliotoxin compared with unexposed mice. Allergen challenge in sensitized mice increased the isoprostane levels compared with control mice, which were further enhanced by exposure to the mycotoxins (Figure 7E).

Increased Allergic Immune Response by Mycotoxins Is Abrogated by the Antioxidant NAC

Because treatment with thiol antioxidants was able to recover the reduced IL-12 production in mycotoxin-exposed DCs

in vitro (Figure 4E), we administered Balb/c mice NAC, a widely used antioxidant and pharmaceutical drug (14, 26), 30 minutes before exposure to gliotoxin or patulin in the acute murine asthma model. The exacerbated asthma-like phenotype in mycotoxin-exposed mice compared with mice that were only sensitized to OVA was diminished by pretreatment with NAC. Similarly, AHR, OVA-specific IgE levels, and the number of eosinophils in the BAL fluid were significantly reduced in NAC-treated mice exposed to mycotoxins (Figures 8A–8D). However, pretreatment of OVA-immunized mice with NAC had no significant effect on eosinophilic inflammation, AHR, and OVA-specific IgE levels.

DISCUSSION

Epidemiological studies suggest that exposure to indoor mold is associated with increased prevalence of respiratory symptoms and asthma, although the underlying mechanisms of the adverse effect

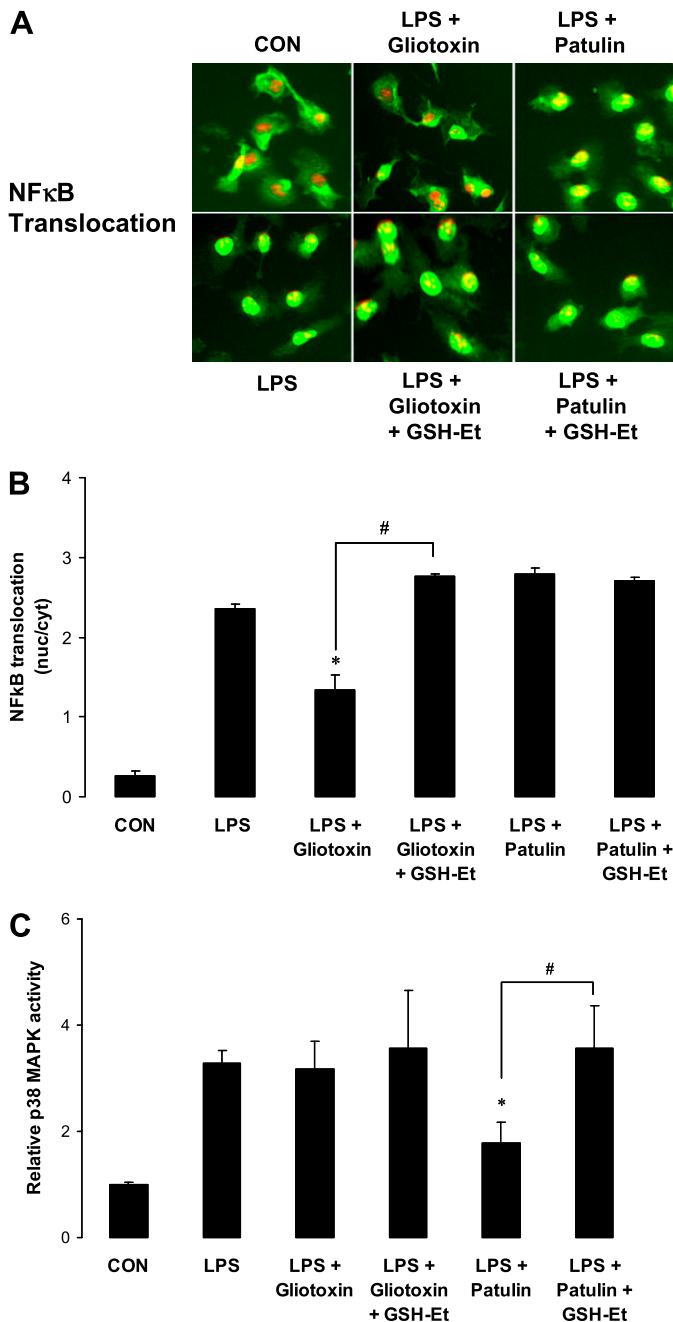


Figure 6. Reduced dendritic cell (DC) function after mycotoxin exposure is mediated by inhibition of nuclear factor (NF)- κ B translocation or p38 mitogen-activated protein kinase (MAPK) activity. (A, B) Exposure of bone marrow-derived DCs to gliotoxin (25 ng/ml) significantly inhibited the lipopolysaccharide (LPS)-induced NF- κ B translocation, whereas patulin treatment (100 ng/ml) did not alter the translocation of NF- κ B. (C) LPS-induced p38 MAPK activity was inhibited after patulin exposure. Data are expressed as mean $\pm$ SEM from three independent experiments (B, C) and representative results are shown (A). * $P < 0.05$, LPS versus LPS + mycotoxin; # $P < 0.05$, LPS + mycotoxin versus LPS + mycotoxin + glutathione ethyl ester (GSH-Et); one-way analysis of variance and Bonferroni multiple comparison test.

remain unclear (27, 28). In the current study, we investigated whether mycotoxins could act as adjuvants of an allergic response. Therefore, on exposure of Balb/c mice to gliotoxin or patulin, we measured their impact in the development of OVA-specific immune responses in established acute and chronic murine

asthma models (15–17). Respiratory exposure to gliotoxin as well as exposure to patulin orally during antigen sensitization of mice clearly increased the asthma-like phenotype in the acute model and enhanced the manifestation of chronic airway inflammation and airway remodeling as well. The observed adjuvant effect by the mycotoxins is comparable to effects caused by exposure to other air pollutants as described in studies for elemental carbon ultrafine particle (EC-UFP) inhalation (29), diesel exhaust (30), environmental tobacco smoke (31), or ozone (32). Furthermore, mycotoxin exposure after OVA immunization and the first antigen challenge also increased airway inflammation and IgE levels, suggesting an adverse health effect in already sensitized individuals. Our results indicate an immune modulatory effect of gliotoxin and patulin during antigen sensitization or antigen challenge, whereas the adjuvant impact on the asthma phenotype was independent of the exposure route, suggesting a more general influence on the immune response.

The World Health Organization has established a provisional maximum tolerable daily intake for patulin of 0.4 μ g/kg of body weight (33). However, it has been shown that children during the first year of life consume more apple products than adults (34) and therefore may also achieve daily patulin intake levels of greater than 1 μ g/kg of body weight (35). After extrapolation from human exposure levels (surface area, variability), as done by Llewellyn and colleagues (35), our applied patulin doses for mice is comparable to real-life exposure conditions, particularly considering that mice received patulin not daily but only six times. Data of relevant gliotoxin concentration after exposure to molds are not available from either human or mouse studies. In the present study we detected adjuvant effects on airway inflammation, the number of eosinophils in the BAL fluid, and on OVA-specific IgG₁ levels of gliotoxin *in vivo* already at 0.1 ng (4 ng/kg, given intranasally six times in a 21-day protocol) that were observable but not significant, and several significantly enhanced asthma parameters at a concentration of 1 ng (number of eosinophils, IgG₁, IL-5, IL-13). Published calculated human maximum inhalation doses for other mycotoxins achieve different concentrations (e.g., 1.4 ng/kg per day for aflatoxin B₂, 5.4 ng/kg per day for satratoxins G+H, and 19 ng/kg per day for aflatoxin B₁) (36). To date we do not know whether gliotoxin concentrations approximate real-world exposures. However, the situation and the conditions in the human lung after daily inhalation of organic dust containing molds or their spores is surely complex, and a predication of possible mycotoxin concentrations *in vivo* is difficult. Compared with other mice studies investigating immunomodulatory effects of gliotoxin, the effective dose in our study was remarkably low (37, 38).

It has been demonstrated that both mycotoxins possess a set of bioactivities. Gliotoxin has been shown to have antibacterial, antiviral, and immunosuppressive properties (37, 39). Patulin was found to exert various effects, including antibacterial effects, disruption of transcription and translation, and inhibition of DNA and protein synthesis (9). Experimental studies using human peripheral blood mononuclear cells have demonstrated *in vitro* that gliotoxin and patulin are able to modulate T-cell function and induce a shift of the Th1/Th2 balance toward Th2 (12, 40). Our *in vivo* study confirmed these results, demonstrated by increased Th2 cytokine levels and a reduced IFN- γ production in mycotoxin-exposed OVA-sensitized mice. These data support the hypothesis of a decreased Th1 differentiation as an explanation for the observed adjuvant effects.

DCs are central to the development of either Th1 or Th2 immunity because cytokine milieu during antigen presentation and recognition are crucial to initiate appropriate immune re-

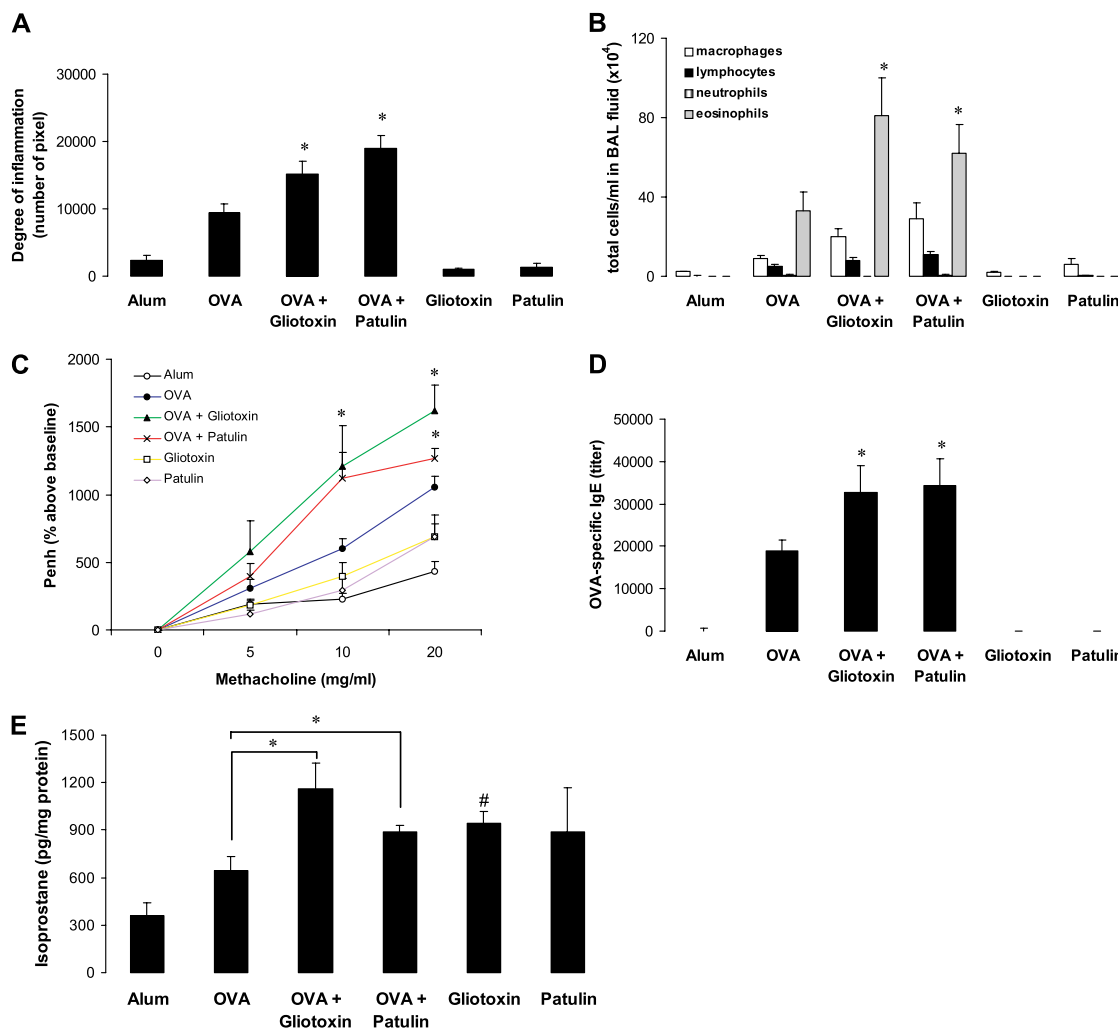


Figure 7. Exposure to mycotoxins during antigen challenge increases airway inflammation, IgE levels, and airway hyper-responsiveness (AHR) and induces an increase in isoprostane levels. To analyze the effect of mycotoxin exposure on an ongoing allergic inflammation, Balb/c mice were sensitized and exposed to the mycotoxins as described under METHODS. To measure 8-isoprostane, lung tissues were prepared as described in METHODS in the online supplement. Effect of exposure to gliotoxin or patulin on (A) inflammation in the lung, (B) total cell numbers in bronchoalveolar lavage (BAL) fluid, (C) AHR, (D) IgE levels, and (E) the production of 8-isoprostane in lung homogenates. Data are expressed as mean $\pm$ SEM, $n \geq 9$ animals per group; * $P < 0.05$ versus ovalbumin (OVA); # $P < 0.05$, gliotoxin versus Alum, one-way analysis of variance and Bonferroni multiple comparison test. Penh = enhanced pause.

sponses and could be responsible for the observed adjuvant effects of mycotoxin exposure in OVA-sensitized mice. Therefore, we investigated the capability of both mycotoxins to alter the Th1/Th2 balance via modification of DC activation. DCs secrete IL-12, which drives IFN- γ production in antigen-specific Th1 cells (21). To induce DC maturation, cells were treated with LPS as shown previously in several studies (41, 42). Treatment of DCs with gliotoxin or patulin inhibited both the LPS-induced IL-12 production and the expression of costimulatory molecules, such as CD80 and CD86. Significantly reduced IL-12 levels were detected at concentrations starting at 6.25 ng/ml gliotoxin and 12.5 ng/ml patulin, respectively. As confirmed by other studies (12, 40), the mycotoxin concentrations of up to 50 ng/ml gliotoxin and 100 ng/ml patulin used by us did not affect cell viability, indicating an influence on immune function by the mycotoxins at subtoxic levels. IL-12 production is an important facet for asthma and allergic disease, because it has been shown that DCs from atopic individuals produce less IL-12 than DCs from healthy individuals, thus favoring Th2 differentiation (43, 44). To show the DC-polarizing effect of mycotoxin exposure on T-cell function, we cocultured purified T cells from DO11.10 mice with purified splenic DCs isolated from mycotoxin-exposed mice or control mice in the presence of OVA. We found that the production of IFN- γ by the transgenic T cells is impaired when Balb/c DC donors had been exposed *in vivo* to the mycotoxins. In a subsequent experiment we transferred OVA-pulsed or additionally mycotoxin-exposed DCs into naive Balb/c mice. Our results

demonstrate that the transfer of mycotoxin-treated and OVA-pulsed DCs enhanced AHR and eosinophilic airway inflammation as well as IL-5 and IL-13 levels and reduced IFN- γ production in the mediastinal lymph nodes compared with mice that received only OVA-pulsed DCs. These data implicate that the reduced IFN- γ production after mycotoxin exposure *in vivo* is specifically due to the effects of mycotoxins on DC populations. A comparable influence on DC function was also demonstrated for diesel exhaust particles (41).

Antigens, such as the experimental allergen OVA, that do not have any intrinsic activating properties need additional signals to trigger DC activation. It has been shown that concomitant exposure of antigens and pathogen-associated molecular patterns such as LPS is required to activate DCs to mount an immune response (45, 46). In our study the used OVA contained traceable amounts of LPS. In mouse models it was demonstrated that low doses of LPS promote asthma development, whereas higher levels appear to be protective (46). The endotoxin contamination of commercial OVA has been shown to suppress AHR and airway inflammation (47). However, the fact that the OVA used in most of the experimental mouse studies contains endotoxin may have direct clinical relevance, because most inhaled allergens, such as allergens derived from cockroaches and house dust mites, are contaminated with LPS (45) or exhibit other pathogen-associated molecular patterns, such as house dust mite-derived β -glucans that bind to non-Toll pattern-recognition receptors (48). However, exposure to my-

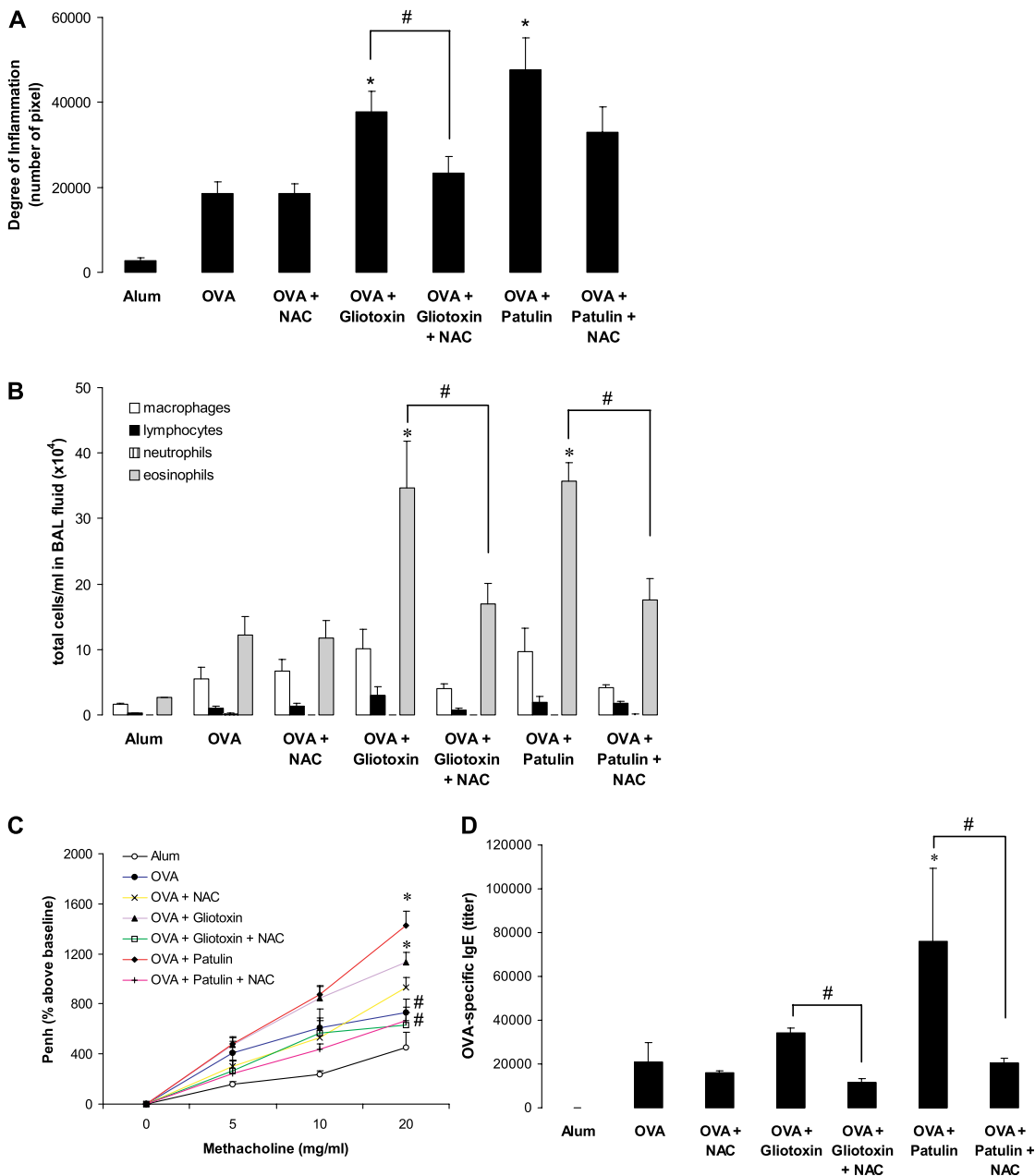


Figure 8. Increased acute allergic immune response by mycotoxins is abrogated by the antioxidant N-acetylcysteine (NAC). Treatment of Balb/c mice with NAC (300 mg/kg intraperitoneally) 30 minutes before exposure to gliotoxin (100 ng) or patulin (20 µg) reduced (A) the exacerbated acute airway inflammation, (B) the number of eosinophils in the BAL fluid, (C) the AHR, and (D) the ovalbumin (OVA)-specific IgE levels compared with NAC-untreated mice. Data are expressed as mean ± SEM, $n \geq 9$ animals per group; * $P < 0.05$, mycotoxins versus OVA; # $P < 0.05$, mycotoxins versus mycotoxins + NAC; one-way analysis of variance and Bonferroni multiple comparison test. Penh = enhanced pause.

cotoxins also increased the allergic immune response in a model using an endotoxin-free OVA, indicating the adjuvant potential of the mycotoxins.

It has been shown previously that intracellular glutathione levels play a central role in determining the Th1/Th2 polarization of DCs, whereby decreased glutathione levels correspond to a Th2 response (22, 49). In addition, the capability of mycotoxins to deplete glutathione could be demonstrated in peripheral blood mononuclear cells and human alveolar epithelial cells (40, 50). Here, treatment of LPS-stimulated DCs with patulin or gliotoxin reduced total glutathione, which may be responsible for the reduced IL-12 production after mycotoxin treatment. Furthermore, pretreatment of mycotoxin-exposed DCs with the thiol antioxidants NAC or GSH-Et abrogated the mycotoxin-caused IL-12 reduction suggesting an involvement of oxidative stress. The modulation of DC and T-cell function by oxidative stress has been shown previously (51).

In our study, we could detect low oxidative stress by 2',7'-dichlorofluorescein-diacetate staining in mycotoxin-treated

DCs measured by flow cytometry (data not shown). The exacerbated asthma phenotype in OVA-sensitized mice exposed to mycotoxins was abolished after pretreatment of mice with the antioxidant NAC, supporting our *in vitro* results. In contrast to other studies showing an involvement of the nuclear erythroid 2 p45-related factor 2 (Nrf2) in the prooxidative diesel exhaust particle-mediated modulation of DC function (41), we could not find Nrf2 translocation in response to mycotoxin exposure (data not shown). Stimulation of DCs with LPS via toll-like receptor 4 activates NF-κB as well as p38 MAP kinase pathways, which are involved in transcriptional activation of genes encoding for maturation receptors and IL-12 (24). Here, we demonstrated a significant reduction of NF-κB translocation by treatment with gliotoxin in LPS-stimulated DCs that was restored by GSH-Et. The capability of gliotoxin to suppress NF-κB activation by inhibition of IκB degradation has been shown in previous studies (52, 53), although the exact molecular mechanisms are still not well established. Interestingly, in our study we could not find an inhibitory effect of patulin on the LPS-stimulated NF-κB trans-

location. In contrast, patulin reduced the phosphorylation of MAPK p38, whereas gliotoxin did not exert a significant effect. It has been already shown for other chemicals, such as sodium methyl dithiocarbamate, that the reduction of the LPS-stimulated IL-12 production via oxidative stress may be mediated by suppression of p38 MAP kinase activation but not by inhibition of NF- κ B activation (54). These data suggest different mechanisms of the structurally different mycotoxins in the molecular signaling leading to the altered DC function.

Furthermore, an increased asthma phenotype after mycotoxin exposure of already sensitized mice implicates an additional mechanism for lung inflammation. Recently it has been shown that exposure to elemental carbon ultrafine particles in mice induced oxidative stress in the airways, measured by 8-isoprostane in lung tissues (25). In the present study, exposure of OVA-sensitized mice during the allergen challenge to mycotoxins enhanced the isoprostane levels compared with nonexposed OVA-sensitized animals. Although exposure of nonsensitized mice to mycotoxins had no effect on asthma parameters or inflammatory cytokines, such as thymic stromal lymphopoietin, tumor necrosis factor- α , or IL-6 in lung tissues, gliotoxin and patulin did induce a moderate increase in isoprostane levels. Our results are comparable with earlier findings (25) and implicate the induction of oxidative stress in the lung by other cell types, such as macrophages or epithelial cells, as an additional mechanism for the observed adjuvant effects of mycotoxin exposure. Mycotoxins may also have an effect on T cells as shown in a previous study demonstrating a reduced IFN- γ production by an IL-12 independent but still unknown mechanism (40). Here, our results show that coculture of isolated CD4⁺ T cells from mycotoxin-exposed DO11.10 mice with CD11⁺ cells from unexposed Balb/c mice significantly decreased IFN- γ production. Mycotoxin exposure may also influence the number or the function of regulatory T cells leading to an increased allergic immune response; however, this remains to be elucidated.

In summary, our data clearly show that exposure of sensitized mice to gliotoxin or patulin increased the asthma-like phenotype. The observed adjuvant effect of gliotoxin and patulin is mediated by affecting the DC function that resulted in reduced IL-12 production in DCs and diminished IFN- γ production in T cells. The decreased Th1 differentiation could be one explanation for the exacerbated allergic immune response after mycotoxin exposure. In addition, exposure to mycotoxins caused oxidative stress in the lung that may have also enhanced the allergic immune response. Taken together, our results suggest that mycotoxins could be responsible for the observed health effects of indoor mold exposure.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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References

- Umetu DT, McIntire JJ, Akbari O, Macaubas C, DeKruyff RH. Asthma: an epidemic of dysregulated immunity. *Nat Immunol* 2002; 3:715–720.
- Leech JA, Nelson WC, Burnett RT, Aaron S, Raizenne ME. It's about time: A comparison of Canadian and American time-activity patterns. *J Expo Anal Environ Epidemiol* 2002;12:427–432.
- Platts-Mills TA, Vervloet D, Thomas WR, Aalberse RC, Chapman MD. Indoor allergens and asthma: report of the Third International Workshop. *J Allergy Clin Immunol* 1997;100:S2–S24.
- Langley SJ, Goldthorpe S, Craven M, Morris J, Woodcock A, Custovic A. Exposure and sensitization to indoor allergens: association with lung function, bronchial reactivity, and exhaled nitric oxide measures in asthma. *J Allergy Clin Immunol* 2003;112:362–368.
- Bunger J, Westphal G, Monnich A, Hinnendahl B, Hallier E, Muller M. Cytotoxicity of occupationally and environmentally relevant mycotoxins. *Toxicology* 2004;202:199–211.
- Jarvis BB, Miller JD. Mycotoxins as harmful indoor air contaminants. *Appl Microbiol Biotechnol* 2005;66:367–372.
- Bennett JW, Klich M. Mycotoxins. *Clin Microbiol Rev* 2003;16:497–516.
- Nieminen SM, Karki R, Auriola S, Toivola M, Laatsch H, Laatikainen R, Hyvarinen A, Von Wright A. Isolation and identification of aspergillus fumigatus mycotoxins on growth medium and some building materials. *Appl Environ Microbiol* 2002;68:4871–4875.
- Moake MMP-ZO, Worobo RW. Comprehensive review of patulin control methods in foods. *Compr Rev Food Sci Food Saf* 2005;4:8–21.
- Ammann HM. Is indoor mold contamination a threat to health? Part two. *J Environ Health* 2003;66:47–49.
- Campbell AW, Thrasher JD, Gray MR, Vojdani A. Mold and mycotoxins: effects on the neurological and immune systems in humans. *Adv Appl Microbiol* 2004;55:375–406.
- Wichmann G, Herbarth O, Lehmann I. The mycotoxins citrinin, gliotoxin, and patulin affect interferon-gamma rather than interleukin-4 production in human blood cells. *Environ Toxicol* 2002;17:211–218.
- Polte T, Schutze N, Lehmann I, Simon J. Mycotoxins exacerbate the allergic immune response in a murine asthma model [abstract]. *Allergy* 2009;64:374.
- Whitekus MJ, Li N, Zhang M, Wang M, Horwitz MA, Nelson SK, Horwitz LD, Brechun N, Diaz-Sanchez D, Nel AE. Thiol antioxidants inhibit the adjuvant effects of aerosolized diesel exhaust particles in a murine model for ovalbumin sensitization. *J Immunol* 2002;168: 2560–2567.
- Polte T, Foell J, Werner C, Hoymann HG, Braun A, Burdach S, Mittler RS, Hansen G. CD137-mediated immunotherapy for allergic asthma. *J Clin Invest* 2006;116:1025–1036.
- Polte T, Hennig C, Hansen G. Allergy prevention starts before conception: maternofetal transfer of tolerance protects against the development of asthma. *J Allergy Clin Immunol* 2008;122:1022–1030.e5.
- Polte T, Behrendt AK, Hansen G. Direct evidence for a critical role of CD30 in the development of allergic asthma. *J Allergy Clin Immunol* 2006;118:942–948.
- Polte T, Fuchs L, Behrendt AK, Hansen G. Different role of CD30 in the development of acute and chronic airway inflammation in a murine asthma model. *Eur J Immunol* 2009;39:1736–1742.
- Lendrum AC, Fraser DS, Slidders W, Henderson R. Studies on the character and staining of fibrin. *J Clin Pathol* 1962;15:401–413.
- Humbles AA, Lloyd CM, McMillan SJ, Friend DS, Xanthou G, McKenna EE, Ghiran S, Gerard NP, Yu C, Orkin SH, et al. A critical role for eosinophils in allergic airways remodeling. *Science* 2004;305: 1776–1779.
- Takeda K, Akira S. TLR signaling pathways. *Semin Immunol* 2004;16:3–9.
- Peterson JD, Herzenberg LA, Vazquez K, Waltenbaugh C. Glutathione levels in antigen-presenting cells modulate Th1 versus Th2 response patterns. *Proc Natl Acad Sci USA* 1998;95:3071–3076.
- Murphy KM, Heimberger AB, Loh DY. Induction by antigen of intrathymic apoptosis of CD4⁺CD8⁺TCR α 0 thymocytes in vivo. *Science* 1990;250:1720–1723.
- Aderem A, Ulevitch RJ. Toll-like receptors in the induction of the innate immune response. *Nature* 2000;406:782–787.
- Alessandrini F, Beck-Speier I, Krappmann D, Weichenmeier I, Takenaka S, Karg E, Kloos B, Schulz H, Jakob T, Mempel M, et al. Role of oxidative stress in ultrafine particle-induced exacerbation of allergic lung inflammation. *Am J Respir Crit Care Med* 2009;179:984–991.
- Dodd S, Dean O, Copolov DL, Malhi GS, Berk M. N-acetylcysteine for antioxidant therapy: pharmacology and clinical utility. *Expert Opin Biol Ther* 2008;8:1955–1962.
- Gent JF, Ren P, Belanger K, Triche E, Bracken MB, Holford TR, Leaderer BP. Levels of household mold associated with respiratory symptoms in the first year of life in a cohort at risk for asthma. *Environ Health Perspect* 2002;110:A781–A786.
- Belanger K, Beckett W, Triche E, Bracken MB, Holford T, Ren P, McSharry JE, Gold DR, Platts-Mills TA, Leaderer BP. Symptoms of wheeze and persistent cough in the first year of life: associations with indoor allergens, air contaminants, and maternal history of asthma. *Am J Epidemiol* 2003;158:195–202.
- Alessandrini F, Schulz H, Takenaka S, Lentner B, Karg E, Behrendt H, Jakob T. Effects of ultrafine carbon particle inhalation on allergic inflammation of the lung. *J Allergy Clin Immunol* 2006;117: 824–830.

30. Miyabara Y, Ichinose T, Takano H, Lim HB, Sagai M. Effects of diesel exhaust on allergic airway inflammation in mice. *J Allergy Clin Immunol* 1998;102:805–812.
31. Min MG, Song DJ, Miller M, Cho JY, McElwain S, Ferguson P, Broide DH. Coexposure to environmental tobacco smoke increases levels of allergen-induced airway remodeling in mice. *J Immunol* 2007;178:5321–5328.
32. Kierstein S, Krytska K, Sharma S, Amrani Y, Salmon M, Panettieri RA Jr, Zangrilli J, Haczku A. Ozone inhalation induces exacerbation of eosinophilic airway inflammation and hyperresponsiveness in allergen-sensitized mice. *Allergy* 2008;63:438–446.
33. Evaluation of certain food additives and contaminants. *World Health Organ Tech Rep Ser* 1995;859:1–54.
34. Plunkett LM, Turnbull D, Rudricks JV. Differences between adults and children affecting exposure assessment. Washington: ILSI Press; 1992.
35. Llewellyn GC, McCay JA, Brown RD, Musgrove DL, Butterworth LF, Munson AE, White KL Jr. Immunological evaluation of the mycotoxin patulin in female B6C3F1 mice. *Food Chem Toxicol* 1998;36:1107–1115.
36. Kelman BJ, Robbins CA, Swenson LJ, Hardin BD. Risk from inhaled mycotoxins in indoor office and residential environments. *Int J Toxicol* 2004;23:3–10.
37. Sutton P, Newcombe NR, Waring P, Mullbacher A. In vivo immunosuppressive activity of gliotoxin, a metabolite produced by human pathogenic fungi. *Infect Immun* 1994;62:1192–1198.
38. Herfarth H, Brand K, Rath HC, Rogler G, Scholmerich J, Falk W. Nuclear factor-kappa b activity and intestinal inflammation in dextran sulphate sodium (DSS)-induced colitis in mice is suppressed by gliotoxin. *Clin Exp Immunol* 2000;120:59–65.
39. Waring P, Beaver J. Gliotoxin and related epipolythiodioxopiperazines. *Gen Pharmacol* 1996;27:1311–1316.
40. Luft P, Oostingh GJ, Gruijthuisen Y, Horejs-Hoeck J, Lehmann I, Duschl A. Patulin influences the expression of Th1/Th2 cytokines by activated peripheral blood mononuclear cells and T cells through depletion of intracellular glutathione. *Environ Toxicol* 2008;23:84–95.
41. Chan RC, Wang M, Li N, Yanagawa Y, Onoe K, Lee JJ, Nel AE. Pro-oxidative diesel exhaust particle chemicals inhibit LPS-induced dendritic cell responses involved in T-helper differentiation. *J Allergy Clin Immunol* 2006;118:455–465.
42. Gutermuth J, Bewersdorff M, Traidl-Hoffmann C, Ring J, Mueller MJ, Behrendt H, Jakob T. Immunomodulatory effects of aqueous birch pollen extracts and phytoprostanes on primary immune responses in vivo. *J Allergy Clin Immunol* 2007;120:293–299.
43. Reider N, Reider D, Ebner S, Holzmann S, Herold M, Fritsch P, Romani N. Dendritic cells contribute to the development of atopy by an insufficiency in IL-12 production. *J Allergy Clin Immunol* 2002;109:89–95.
44. Chen XQ, Yang J, Hu SP, Nie HX, Mao GY, Chen HB. Increased expression of CD86 and reduced production of IL-12 and IL-10 by monocyte-derived dendritic cells from allergic asthmatics and their effects on Th1- and Th2-type cytokine balance. *Respiration* 2006;73:34–40.
45. Willart MA, Lambrecht BN. The danger within: endogenous danger signals, atopy and asthma. *Clin Exp Allergy* 2009;39:12–19.
46. Eisenbarth SC, Piggott DA, Huleatt JW, Visintin I, Herrick CA, Bottomly K. Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. *J Exp Med* 2002;196:1645–1651.
47. Watanabe J, Miyazaki Y, Zimmerman GA, Albertine KH, McIntyre TM. Endotoxin contamination of ovalbumin suppresses murine immunologic responses and development of airway hyper-reactivity. *J Biol Chem* 2003;278:42361–42368.
48. Nathan AT, Peterson EA, Chakir J, Wills-Karp M. Innate immune responses of airway epithelium to house dust mite are mediated through beta-glucan-dependent pathways. *J Allergy Clin Immunol* 2009;123:612–618.
49. Murata Y, Ohteki T, Koyasu S, Hamuro J. IFN-gamma and pro-inflammatory cytokine production by antigen-presenting cells is dictated by intracellular thiol redox status regulated by oxygen tension. *Eur J Immunol* 2002;32:2866–2873.
50. Johannessen LN, Nilsen AM, Lovik M. Mycotoxin-induced depletion of intracellular glutathione and altered cytokine production in the human alveolar epithelial cell line A549. *Toxicol Lett* 2007;168:103–112.
51. Kantengwa S, Jornt L, Devenoges C, Nicod LP. Superoxide anions induce the maturation of human dendritic cells. *Am J Respir Crit Care Med* 2003;167:431–437.
52. Pahl HL, Krauss B, Schulze-Osthoff K, Decker T, Traenckner EB, Vogt M, Myers C, Parks T, Warring P, Muhlbacher A, *et al.* The immunosuppressive fungal metabolite gliotoxin specifically inhibits transcription factor NF- κ B. *J Exp Med* 1996;183:1829–1840.
53. Wilson SJ, Leone BA, Anderson D, Manning A, Holgate ST. Immunohistochemical analysis of the activation of NF- κ B and expression of associated cytokines and adhesion molecules in human models of allergic inflammation. *J Pathol* 1999;189:265–272.
54. Pruett SB, Zheng Q, Schwab C, Fan R. Sodium methylthiocarbamate inhibits map kinase activation through toll-like receptor 4, alters cytokine production by mouse peritoneal macrophages, and suppresses innate immunity. *Toxicol Sci* 2005;87:75–85.

Exposure to mycotoxins increases the allergic immune response in a murine asthma model

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ONLINE DATA SUPPLEMENT

METHODS

Mice

Mice were bred and maintained in animal facility at the University of Leipzig (Germany) and housed under conventional conditions with 23°C room temperature, 60% humidity, and 12 h day/night rhythm. Cages were bedded with LIGNOCEL® bedding material (fine particles < 200 µm 0.2%). The mice received conventional mouse feed (Altromin, Lage, Germany) and water *ad libitum*. All animal experiments involved groups of 4-6 mice/cage and were performed at least 2 times.

Mycotoxins

The mycotoxins used for this study are different in their chemical structure. Gliotoxin belongs to the epipolythiodioxopiperazine class of secondary metabolites, characterized by the bridged disulphide piperazidone six-membered ring that appears to be necessary for the most of the biological properties of these compounds (1). Patulin has an ylidenebutenolide structure. Gliotoxin (Sigma-Aldrich, Steinheim, Germany) is obtained by *Gliocladium fimbriatum*, patulin (Sigma-Aldrich) by *Penicillium fumigatus*.

Collection of bronchoalveolar lavage (BAL) fluid

Cells in the lavage fluid were counted using a hemocytometer and BAL cell differentials were determined as described previously (2, 3).

Lung histology and computer-based quantification of inflammation, mucus production, extracellular matrix deposition and airway smooth muscle proliferation. Left lung was fixed in 10% formalin and stained with Haematoxylin & Eosin (H&E, MERCK, Darmstadt, Germany) or with Alcian blue-PAS (Sigma-Aldrich).

For quantification and objective evaluation of the degree of histological inflammation and mucus production, lung sections were scanned with a digital camera (Zeiss, 5 shots per lung) and analyzed with HistoClick-Software (provided by Ingo Hohmann and Wiebke Scherf) based on morphometric image analysis (2, 4). The degree of inflammation is expressed by the number of pixels which correlate to the stained cells of interest. Total matrix deposition was assessed on Martius Scarlet Blue (MSB)-stained sections (5). Lung sections were also immunostained with anti- α -smooth muscle actin (SMA) primary Ab to distinguish smooth muscle cells and with proliferating cell nuclear antigen (PCNA) to stain proliferating smooth muscle cells (4). The primaries Abs were detected with a HRP-labeled secondary Ab (both Abcam, USA). Digital photographs of four bronchioles per tissue section were taken and analyzed with HistoClick-Software.

Collagen Analysis. Collagen content was measured in lung tissue homogenates by a biochemical assay according to the manufacturer's instructions (Sircol collagen assay, Biocolor, Ireland). Lung tissue (100 mg) was homogenized in 1 ml Tris buffer containing 1 M sodium chloride and a protease inhibitor cocktail (Sigma-Aldrich, Taufkirchen, Germany). Samples were incubated overnight at 4°C with stirring, centrifugated and the supernatant was assayed (4).

OVA-specific IgE and IgG₁ assay

OVA-specific IgE and IgG₁ serum levels were measured by sandwich ELISA according to a standard protocol as described previously (2).

Cytokine production

Splenocytes or mediastinal lymph node cells (5×10^6 cells/ml per well) were isolated and re-stimulated in vitro with 200 µg/ml OVA in culture medium (RPMI medium supplemented with 10% FCS, 100 U/ml Penicilin, 100 µg/ml Streptomycin) one day after airway function test. Lung tissues were homogenized in PBS and protein was measured at 700 nm with 5 µl homogenate and 200 µl of 1:5 diluted Biorad solution (Bio-Rad, München, Germany) with bovine serum albumin as standard.

Cytokines were measured in supernatants from re-stimulated spleen cells after three days or from lung tissues using DuoSet® ELISA kits (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions.

Measurement of 8-isoprostane

18 hours after the last mycotoxin exposure lung tissues were homogenized in buffer (0.1 M phosphate, 1 mM EDTA, 10 µM indomethacin and 0.05% butylated hydroxytoluene, BHT at pH 7.4) using lysing matrix A (FastPrep®24 homogenizer, MP Biomedicals, LLC, Eschwege, Germany). Aliquots of the obtained supernatants were hydrolysed (15% KOH) and deproteinized (ethanol containing 0.01% BHT). 8-isoprostane concentration was determined by specific immunoassay according to manufacturer's instructions (Cayman Chemical Company, Ann Arbor, MI, USA). Protein concentration of 5 µl homogenates were measured at 700 nm using DC Protein Assay (Bio-Rad, München, Germany) comparing it to diluted series of bovine serum albumin as standard.

Culture, treatment and transfer of bone marrow-derived dendritic cells (BMDC)

Bone marrow cells were collected from naïve mice, depleted of red blood cells using ammonium chloride, and grown in RPMI medium containing 10% FCS and 20 ng/ml rmGM-CSF for 7 days. Cells were characterized by staining with an anti-CD11c–

Phycoerythrin (PE) mAb and 2×10^6 cells/ml were given to 24-well plates and exposed to gliotoxin (6.25-50 ng/ml) or patulin (12.5-100 ng/ml) in the presence of LPS (1 μ g/ml) for 18 hrs. In some experiments, NAC (2 mM) and glutathione ethyl ester (GSH-Et, 0.4 mM, both Sigma-Aldrich) were given to the cells during mycotoxin exposure. The supernatant was collected for cytokine measurement and cells were prepared for analysis of intracellular glutathione.

Total GSH measurement

DCs were exposed to gliotoxin (6.25-25 ng/ml) or patulin (25-100 ng/ml) in the presence of LPS for 18 hrs and total cellular glutathione (GSH) was determined according to the manufacturer's instructions (Sigma-Aldrich). Briefly, cells were washed with PBS, pelleted and resuspended in PBS with sulfosalicylic acid. The cell suspension was frozen and thawed twice followed by centrifugation at 10000 g for 10 min. Samples were diluted 1:5 and glutathione content of the sample was assayed using a kinetic assay in which catalytic amounts of glutathione cause a reduction of 5,5'-dithiobis-(2-nitrobenzoic) acid (DTNB) to TNB. The oxidized glutathione formed is recycled by glutathione reductase and NADPH. The product, TNB, is assayed colorimetrically at 405 nm.

Co-culture of spleen DC and CD4⁺ T cell

Balb/c mice were exposed to gliotoxin (100 ng i.n.) and patulin (20 μ g, days 0 and 1) and OVA-immunized (day 1). The next day splenocytes from exposed and unexposed mice were isolated, pooled and DCs were separated by magnetic cell sorting (Pan DC MicroBeads, Milteny Biotek, Bergisch-Gladbach, Germany, > 90% CD11c⁺ cells). 2.5×10^5 spleen DCs were co-cultured with separated CD4⁺ T cells (>

95%, 2.5×10^5 cells, final volume 1 ml) from DO11.10 mice and stimulated with OVA (20 μ g) for 24 h (IL-12) and 48 h (IL-5, IL-13, IFN- γ) to measure cytokine production in the supernatant (6).

Transfer of OVA-pulsed DCs

Immunization by intrapulmonary injection of OVA-pulsed DCs was done as described Lambrecht *et al.* (7, 8). Briefly, bone marrow-derived DCs were pulsed for 24 h with OVA (200 μ g/ml), some cells in the presence of gliotoxin (25 ng/ml) or patulin (100 ng/ml). 2×10^6 cells in 80 μ l PBS were adoptively transferred into the trachea in anesthetized naïve Balb/c mice. From day 10 onward, mice were challenged i.n. with OVA for 3 consecutive days. AHR was measured on day 13 and mice were sacrificed the following day.

NF κ B translocation

BMDCs (0.5×10^5 /well) were exposed to gliotoxin (25 ng/ml) or patulin (100 ng/ml) followed by stimulation with LPS (1 μ g/ml) in 96-well Imaging Plates (BD Falcon™) and fixed (3.7 % formaldehyde/PBS) after 1 hr. Cells were permeabilized in 0.1% TritonX100/PBS for 15 min and washed two times with PBS. NF κ B was localized by rabbit-anti-NF κ B p65 IgG (1:100; Santa Cruz Biotechnology, USA) and anti-rab-IgG-Alexa Fluor 647, Fab-fragment (1:400; Invitrogen). To localize nuclei cells were additionally stained with Hoechst 33342 (Invitrogen). The imaging plates were measured by BD Pathway™ 855 High-Content Bioimager. Images were analyzed with attovision software (BD Biosciences). Data represents normalized ratio (intensity nucleus/intensity cytoplasm).

Western blot analysis for p38 MAP kinase activation

BMDCs (2×10^6 /well) were exposed to gliotoxin (25 ng/ml) or patulin (100 ng/ml), in part in the presence of GSH-Et, followed by stimulation with LPS for 1 hr and western blots were done as described previously (9).

Flow cytometric analysis

Flow cytometric analysis was performed using PE- or PerCP-Cy5.5-conjugated mAb to mouse CD11c, CD80, CD86 and I-A/I-E or isotyp-matched control mAb (BD Biosciences Pharmingen, Heidelberg, Germany). 1×10^6 BMDCs were preincubated with 2.4G2 mAb (10 μ g/ml) to block Fc γ R, and then incubated with the relevant mAbs for 15 min. at 4°C. Cells were washed twice with 1% BSA in PBS. Cells were analysed by FACScalibur (BD Biosciences Pharmingen, Heidelberg, Germany).

Figure legends

Figure E1: Effect of mycotoxin exposure on mucus production, antigen-specific IgG1 and cytokine production in lung cells in OVA-immunized mice.

Balb/c mice were immunized with OVA (day 1 and 14) and then challenged with OVA on days 14 and 17-19. Mice were exposed to mycotoxins and challenged with OVA on days 0, 1, 13, 14, 17 and 18. Effect of mycotoxin exposure on mucus production, quantified by an objective, investigator-independent computer analysis (A) OVA-specific IgG₁ levels (B) and cytokine levels in the supernatant of OVA-re-stimulated lung cells (C). Data are expressed as mean $\pm$ SEM, $n \geq 9$ animals per group; $*P < 0.05$, one-way ANOVA and Bonferroni's multiple comparison test.

Figure E2: Mycotoxin exposure of LPS-stimulated DCs suppresses CD80 and CD86 expression.

BMDCs were exposed to mycotoxins and stimulated with LPS for 18 hrs. Flow cytometric analyses were assessed by FACScan. Exposure to gliotoxin (25 ng/ml) and patulin (100 ng/ml) suppressed CD80 (A) and CD86 (B) expression. Data are expressed as mean $\pm$ SEM from three independent experiments. * P < 0.05, one-way ANOVA and Bonferroni's multiple comparison test.

Figure E3: Co-culture of CD4⁺ T cells from mycotoxin-exposed DO11.10 mice with CD11⁺ cells from unexposed Balb/c mice significantly decreased IFN- γ .

Splenic DCs isolated from immunized, un-exposed Balb/c mice were co-cultured with CD4⁺ T cells isolated from mycotoxin-exposed DO11.10 mice and stimulated with OVA. IFN- γ , IL-13 and IL-5 levels are shown. * P < 0.05, CD4⁺ unexposed vs. CD4⁺ exposed; one-way ANOVA and Bonferroni's multiple comparison test.

Figure E4: Effect of mycotoxin exposure in OVA-stimulated DCs on IL-12 production *in vitro* and cytokine production *in vivo* after DC transfer into Balb/c mice.

DCs were pulsed for 24 h with OVA from Sigma (OVA (S), 200 μ g/ml) or with Endograde-OVA (OVA (EG), 200 μ g/ml), some cells in the presence of gliotoxin (25 ng/ml) or patulin (100 ng/ml). As control DCs were treated only with LPS (100 ng/ml). Afterwards DCs were transferred into the trachea of naïve Balb/c mice. From day 10 onward, mice were challenged i.n. with OVA for 3 consecutive days. IL-12 production of DCs before transfer (A) and effect of DC transfer on cytokine production in lung cells (B). Data are expressed as mean $\pm$ SEM; n= 4-5/group. * P < 0.05, LPS or OVA (S) vs. Medium; [#] P < 0.05, mycotoxin + OVA (S) vs. OVA- (S); one-way ANOVA and Bonferroni's multiple comparison test.

Figure E5: Effect of mycotoxin exposure to naïve or immunized Balb/c mice on TSLP, TNF- α , IL-6 or IL-25 levels in lung tissue.

18 hours after the last mycotoxin exposure lung tissues were homogenized in PBS and cytokine levels were measured in the supernatant. Mycotoxin exposure to naïve or OVA-immunized Balb/c mice had no effect on TSLP, TNF- α , IL-6 or IL-25 levels in lung tissue. * $P < 0.05$ vs. OVA, one-way ANOVA and Bonferroni's multiple comparison test.

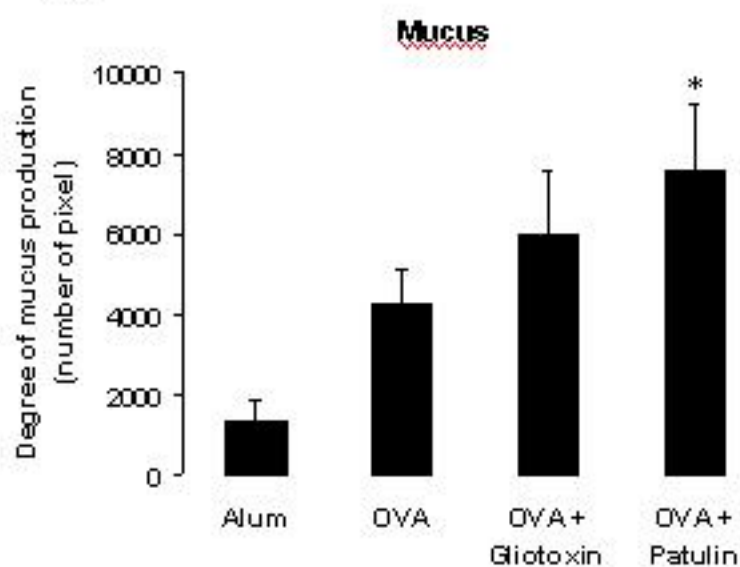
References

- E1. Waring, P., and J. Beaver. 1996. Gliotoxin and related epipolythiodioxopiperazines. *Gen Pharmacol* 27(8):1311-6.
- E2. Polte, T., J. Foell, C. Werner, H. G. Hoymann, A. Braun, S. Burdach, R. S. Mittler, and G. Hansen. 2006. CD137-mediated immunotherapy for allergic asthma. *J Clin Invest* 116(4):1025-1036.
- E3. Polte, T., C. Hennig, and G. Hansen. 2008. Allergy prevention starts before conception: maternofetal transfer of tolerance protects against the development of asthma. *J Allergy Clin Immunol* 122(5):1022-1030 e5.
- E4. Polte, T., L. Fuchs, A. K. Behrendt, and G. Hansen. 2009. Different role of CD30 in the development of acute and chronic airway inflammation in a murine asthma model. *Eur J Immunol* 39(7):1736-42.
- E5. Humbles, A. A., C. M. Lloyd, S. J. McMillan, D. S. Friend, G. Xanthou, E. E. McKenna, S. Ghiran, N. P. Gerard, C. Yu, S. H. Orkin, and C. Gerard. 2004. A critical role for eosinophils in allergic airways remodeling. *Science* 305(5691):1776-9.

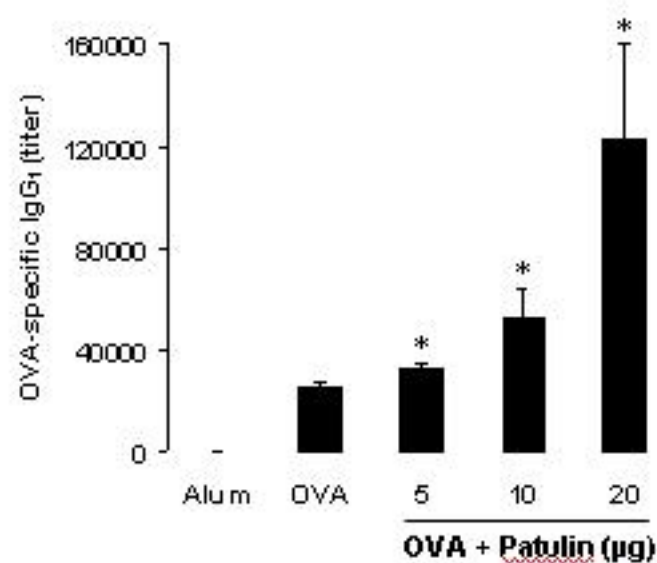
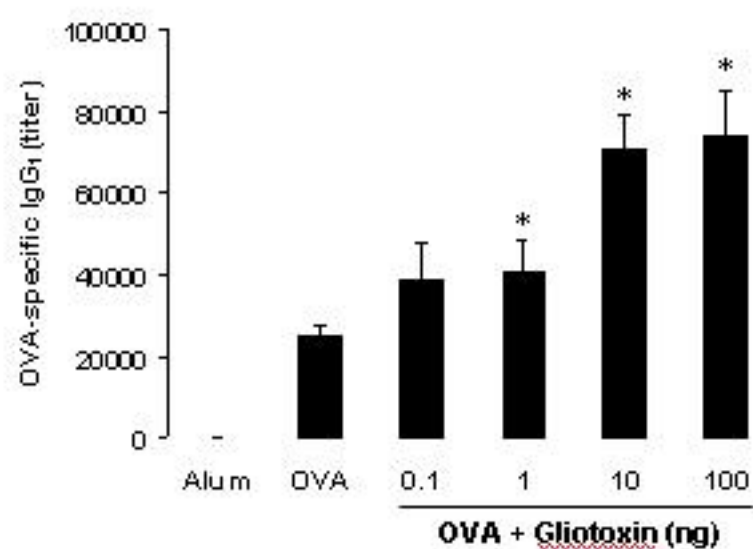
- E6. Peterson, J. D., L. A. Herzenberg, K. Vasquez, and C. Waltenbaugh. 1998. Glutathione levels in antigen-presenting cells modulate Th1 versus Th2 response patterns. *Proc Natl Acad Sci U S A* 95(6):3071-6.
- E7. Lambrecht, B. N., R. A. Pauwels, and B. Fazekas De St Groth. 2000. Induction of rapid T cell activation, division, and recirculation by intratracheal injection of dendritic cells in a TCR transgenic model. *J Immunol* 164(6):2937-46.
- E8. Kuipers, H., T. Soullie, H. Hammad, M. Willart, M. Kool, D. Hijdra, H. C. Hoogsteden, and B. N. Lambrecht. 2009. Sensitization by intratracheally injected dendritic cells is independent of antigen presentation by host antigen-presenting cells. *J Leukoc Biol* 85(1):64-70.
- E9. Roder-Stolinski, C., G. Fischader, G. J. Oostingh, K. Eder, A. Duschl, and I. Lehmann. 2008. Chlorobenzene induces the NF-kappa B and p38 MAP kinase pathways in lung epithelial cells. *Inhal Toxicol* 20(9):813-20.

Figure E1

A



B



C

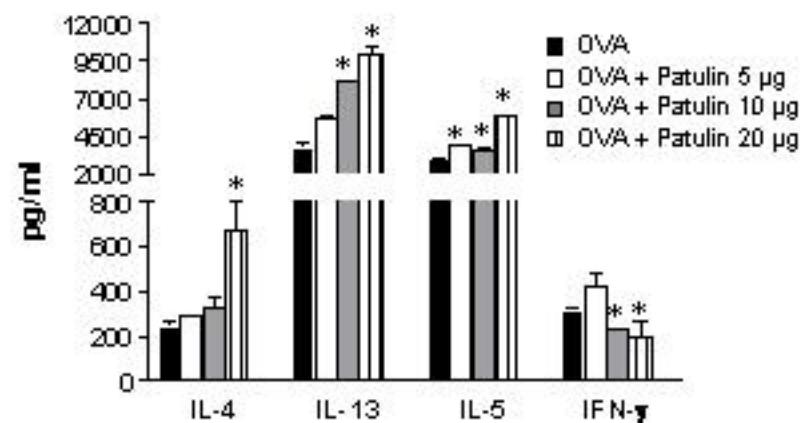
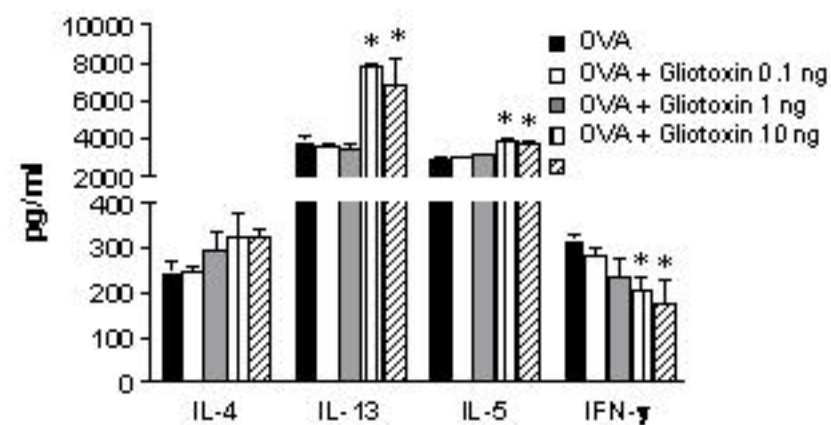


Figure E2

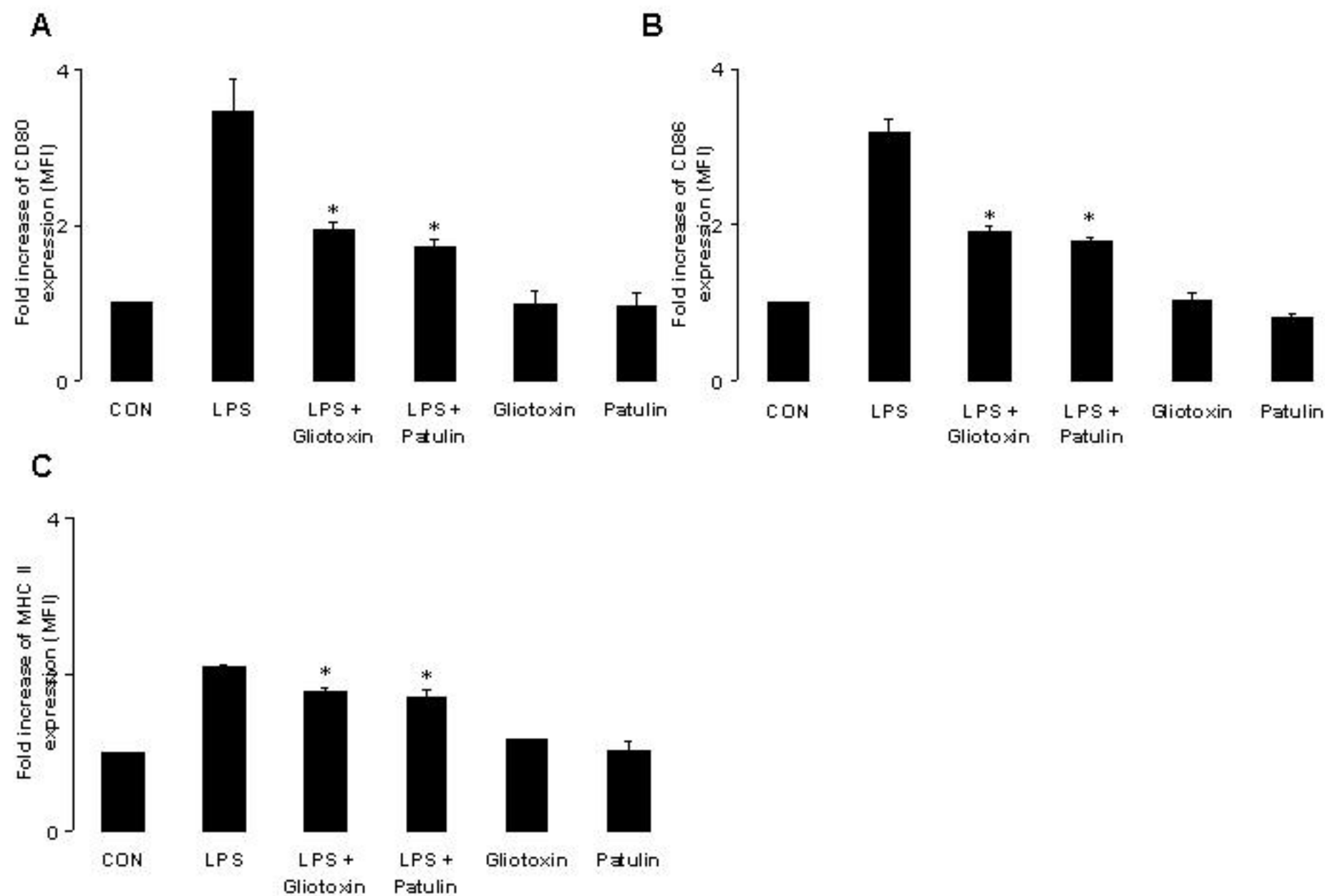


Figure E3

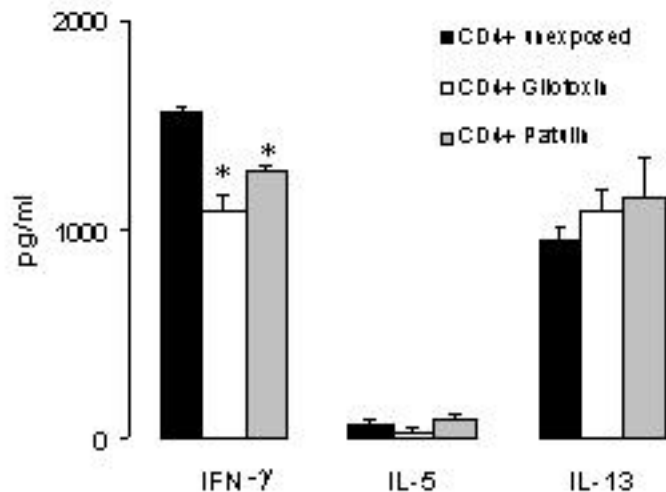


Figure E4

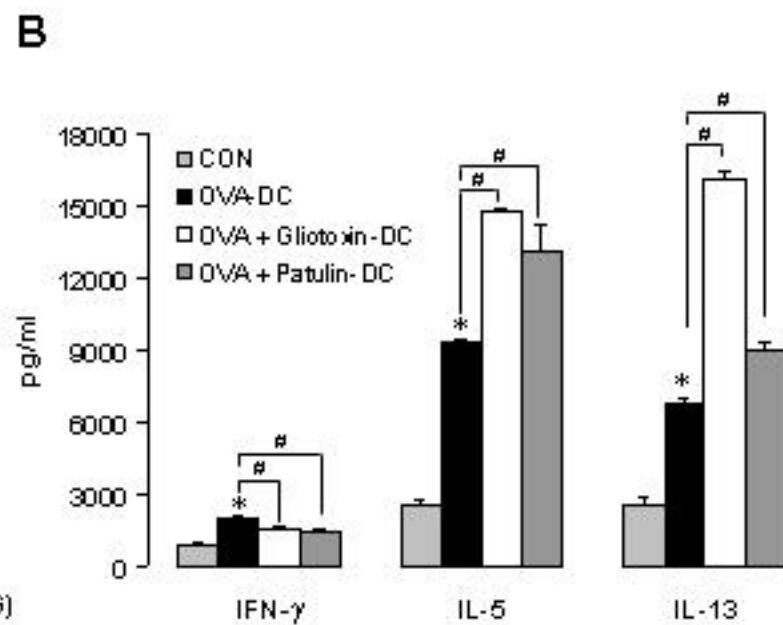
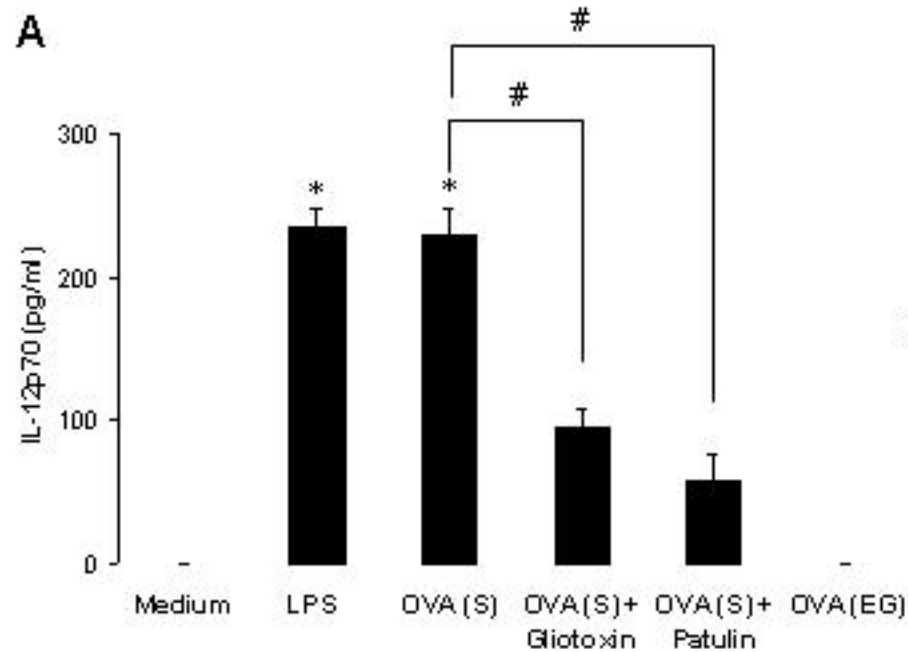


Figure E5

