

Intranasal Exposure to *Stachybotrys chartarum* Enhances Airway Inflammation in Allergic Mice

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Rationale: Exposure to building dampness, often associated with growth of microbes such as *Stachybotrys chartarum*, has been linked to respiratory symptoms. We have shown previously in a murine model that exposure to *S. chartarum* can induce lung inflammation characterized by infiltration of neutrophils and lymphocytes; this process is regulated by proinflammatory cytokines and leucocyte-attracting chemokines.

Objectives: Because an atopic predisposition may influence the response to microbes, we examined the effects of *S. chartarum* on allergic mice in an experimental model. BALB/c mice were sensitized to ovalbumin by intraperitoneal injections and exposed for 3 wk to spores of *S. chartarum*.

Measurements and Main Results: Numbers of eosinophils and neutrophils were drastically increased in bronchoalveolar fluid from these mice as compared with the ovalbumin-sensitized/challenged mice or those exposed to *S. chartarum* without ovalbumin sensitization. Histologic sections showed severe granulomatous inflammatory cell infiltrates in all compartments of the lung, including peribronchial, perivascular, and alveolar spaces. The mRNA levels of proinflammatory cytokines interleukin-1 β and tumor necrosis factor α and the chemokine CCL3/MIP-1 α were also markedly increased in the lungs. Despite the enhancement of the pulmonary inflammatory reaction, exposure to *S. chartarum* spores significantly down-regulated airway hyperresponsiveness and showed a tendency to decrease levels of Th2 cytokines in the lung.

Conclusion: Exposure to *S. chartarum* modulates the inflammatory reaction and airway hyperresponsiveness, depending on the allergic status of the exposed mice.

Keywords: allergy; asthma; experimental model; mold

Airway hyperresponsiveness (AHR), eosinophilia, and increased levels of Th2-type cytokines (e.g., interleukin [IL]-4, IL-5, and IL-13) in the lungs together with the production of serum IgE antibodies are characteristics of allergic asthma (1). These features of the disease can be induced in a murine model of acute allergic asthma, which has been widely used to study the development and treatment of asthma (2).

Acute or chronic exposure to allergens or air pollutants and upper respiratory tract infections affects the airways and influences the severity of asthmatic symptoms. Epidemiologic studies have

shown that exposure to molds has an adverse effect on asthmatic symptoms and bronchial responsiveness (3, 4), with this relationship being more pronounced in individuals sensitized to molds (5–7). In addition to allergy, nonallergic effects of fungal spores and their secondary metabolites have been claimed to play a role in the diverse airway symptoms linked to mold spore exposure (8). Cell wall components, such as (1 $\rightarrow$ 3)- β -D-glucans (9), and the mycotoxins produced by some of the fungal species (10) have been potent inducers of inflammatory airway responses in experimental models.

Stachybotrys chartarum is a damp building mold that can be found in large quantities in water-damaged buildings. Many studies have suggested that exposure to this microbe may have hazardous effects on health. Exposure to *S. chartarum* was linked to serious symptoms, including hemorrhage in the lungs, in infants in Cleveland, Ohio, in the 1990s (11). This association was later questioned (12). The potential to cause adverse health effects may be due to the ability of the microbe to produce mycotoxins (13). We and others have shown that instillations of *S. chartarum* spores into rat or mice lungs can induce airway inflammation (14, 15).

The symptoms associated with human mold spore exposure are diverse, and the health effects associated with exposure to damp building molds may depend on the immunologic status of the exposed individual. To study this phenomenon, we exposed allergic or nonallergic mice intranasally to *S. chartarum*.

METHODS

Animals

Female BALB/cJBom mice, 6 to 10 wk of age, were obtained from M&B Taconic (Ry, Denmark). Mice transgenic for the ovalbumin (OVA)_{323–339}-specific DO11.10 T-cell receptor (OVA-TCR) were from Charles River Laboratories (Sulzfeld, Germany). The mice were housed in specific pathogen-free facilities and maintained on an OVA-free diet. All experimental protocols were approved by the Social and Health Care Department at the State Provincial Office of Southern Finland.

Microbe Preparations

The *S. chartarum* strain s. 72 (NRRL 6084) has been characterized (15) and was grown on rice flour agar as previously described (16). When grown on rice agar, it produces satratoxin G and H, stachybotrylactone, and stachybotrylactam in the amounts of 4 ng, 10 ng, 8 μ g, and 2 μ g, respectively, per 10⁵ spores. The spores were suspended directly from the agar plates into phosphate-buffered saline (PBS) and γ -irradiated with 10 kGy before use.

Sensitization, Airway Challenge, and Intranasal Instillations of *S. chartarum* Spores

Mice were sensitized intraperitoneally on Days 0 and 10 with OVA or sham sensitized with PBS emulsified in alum (17) according to the protocol shown in Figure 1. The intranasal instillation of *S. chartarum* spores has previously been optimized to 2 $\times$ 10⁵ spores in 50 μ l PBS (the controls received PBS only), and the spore suspension was administered under light anesthesia (Isoflurane; Abbott Laboratories Ltd., Queenborough, UK) twice a week starting on Day 2 (16). Eight mice

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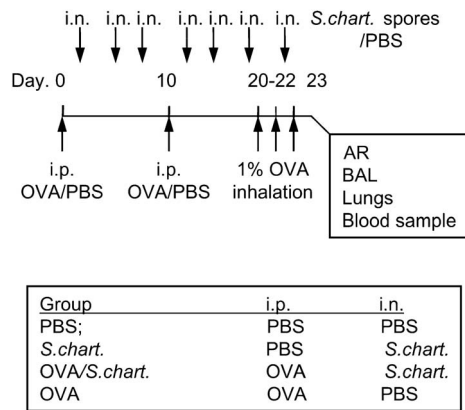


Figure 1. Study protocol. Mice were sensitized on Days 0 and 10 with ovalbumin (OVA) in alum and exposed intranasally to *S. chartarum* spores (OVA-*S. chart.* group) or phosphate-buffered saline (PBS; OVA group) as described in METHODS. For comparison, mice were sham sensitized with PBS and exposed intranasally to *S. chartarum* spores (*S. chart* group) or PBS (PBS group). On Days 20–22, the mice were challenged with aerosolized OVA, 20 min/d. Airway responsiveness (AR) was measured 24 h after the last airway challenge, and specimens were collected. BAL = bronchoalveolar lavage.

($n = 8/\text{group}$) were treated with each administration, and the experiments were repeated twice. On Days 20, 21, and 22, all the mice were challenged with 1% OVA via the airways using an ultrasonic nebulizer (Aerogen Ltd., Galway, Ireland). On Day 23, the airway responsiveness was assessed, and specimens were collected.

Determination of Airway Responsiveness

Airway responsiveness was assessed using single-chamber, whole-body plethysmography (Buxco, Troy, NY). Lung reactivity parameters were recorded at baseline and after increasing concentrations of nebulized methacholine as previously described (16).

Sample Collection and Lung Preparations

The mice were killed, a blood sample was collected, and the lungs were lavaged as described previously (16). The bronchoalveolar lavage fluid (BALF) was stained with May Grünwald-Giemsa stain, and the cells were counted. The supernatant was stored at -70°C for cytokine analysis. The remaining cells were fixed in ethanol (1:2). One part of the left lung was removed for RNA isolation, and the other was embedded into Tissue-Tek O.C.T. compound (Sakura Finetek Europe B.V., Zoeterwoude, The Netherlands) and quick-frozen. The lung samples were cut into 5- μm sections and stained immunohistochemically with monoclonal antibodies against CD3, CD4, and CD8 (BD Biosciences Pharmingen, San Jose, CA) using the ChemMate (DakoCytomation, Glostrup, Denmark) staining kit. The number of positively stained cells was counted in three lymphocytic infiltrates per lung containing at least 25 CD3⁺ cells. The right lung was perfused with 10% formalin and excised en bloc. The lungs were embedded in paraffin, cut, and affixed onto microscope slides. The slides were deparaffinized and stained with hematoxylin and eosin (H&E) and with periodic acid Schiff (PAS) stain and examined under light microscopy (18).

Preparation and *In Vitro* Stimulation of Splenocytes from OVA-specific TCR Transgenic Mice

Mice transgenic for the OVA-specific TCR were sensitized by intraperitoneal injection of 10 μg OVA adsorbed to 0.5 mg alum on Days 0, 14, and 21. On Day 22, single-cell suspensions of spleen cells of naive and sensitized mice were prepared in complete Roswell Park Memorial Institute 1640 medium (Gibco; Invitrogen Corp., Paisley, UK) supplemented by 5% fetal bovine serum, 1 mM sodium pyruvate, 2 mM L-glutamine, 0.05 mM 2-ME, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin. Cells were cultured at 3×10^6 in the medium in 24-well plates

in the presence of OVA (50 $\mu\text{g}/\text{ml}$) or *S. chartarum* spores (10^5). Cells were collected, and total RNA was extracted after 6 h of culture.

Real-Time Quantitative Reverse Transcriptase–Polymerase Chain Reaction Assay

Total RNA from lungs and splenocytes was extracted and transcribed into cDNA (16). Real-time quantitative polymerase chain reaction (PCR) was performed with an AbiPrism 7700 Sequence Detector System (Applied Biosystems, Foster City, CA). PCR primers and probes were obtained as predeveloped assay reagents (IL-1 β , tumor necrosis factor [TNF]- α , IL-4, IL-5, IL-13, IL-12p40, macrophage inflammatory protein [MIP]-1 α , monocyte chemoattractant protein [MCP]-1, MIP-2, and 18 S rRNA) or were generated (IFN- γ , eotaxin-1, IL-18, CCR3, CCR5, and CXCR2) by PrimerExpress version 1.5 software and were ordered from and checked by Applied Biosystems. Endogenous 18S rRNA was used as the housekeeping gene, and the target gene expression was expressed as relative quantities (16).

Measurements of Cyto- and Chemokines in BALF

Cytokine and chemokine levels in BALF were measured with commercial kits according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). The detection limits were less than 5.1 pg/ml for TNF- α and 1.5 pg/ml for CCL3 and IL-13.

Serum Antibody Measurements

Total IgE, OVA, and *S. chartarum*-specific IgE, IgG1, and IgG2a serum levels were measured by ELISA (16).

Statistical Analysis

Data were analyzed with the GraphPadPrism software (GraphPad Software, Inc., San Diego, CA). Differences between groups were analyzed by nonparametric analysis of variance and the Kruskal-Wallis test. Single-group comparisons were performed by the Mann-Whitney test. The results are expressed as mean $\pm$ SEM, and p values less than 0.05 were considered statistically significant.

RESULTS

S. chartarum Spores Induce a Severe Lung Inflammation in OVA-sensitized and OVA-challenged Mice

Total cell number was markedly higher in BALF from the OVA-sensitized/challenged mice exposed intranasally to *S. chartarum* spores compared with the OVA-sensitized/challenged mice or with the mice exposed to the *S. chartarum* (Table 1). Eosinophils predominated in the OVA-sensitized/challenged mice, whereas in the *S. chartarum*-exposed mice, only a mild inflammation was seen in which macrophages predominated. In the OVA-sensitized/challenged mice exposed to *S. chartarum*, a major influx of eosinophils, neutrophils, and lymphocytes was seen. The number of cells was synergistically increased when compared with any of the models separately.

Severe infiltration of inflammatory cells in OVA-sensitized/challenged mice exposed to *S. chartarum* was observed in the H&E-stained lung section (Figure 2C). In the *S. chartarum*-exposed mice, inflammatory cell clusters were seen in the lung tissue, but the overall degree of inflammation was mild (Figures 2B and 2I). In the OVA-sensitized/challenged mice, the inflammatory cells were mostly seen in peribronchial areas. In OVA-sensitized/challenged mice exposed to *S. chartarum*, a severe inflammation was seen in the peribronchial and perivascular areas and in the intervening lung parenchyma. In addition to typical inflammatory cells, significant amounts of multinucleated giant cells and loose epithelioid granulomas were observed within the inflammatory clusters (Figures 2K and 2L). In some bronchioles, the epithelial surface was ulcerated, and granulomatous tissue was seen in the lumen.

Immunohistochemical staining of the lungs showed a decreased CD4/CD8 ratio in OVA-sensitized/challenged mice exposed to

TABLE 1. CELLULAR COMPOSITION OF THE BRONCHOALVEOLAR LAVAGE FLUID

Group	Total Cells (million cells/L)	Eosinophils, million cells/l (%)	Neutrophils, million cells/l (%)	Lymphocytes, million cells/l (%)	Macrophages, million cells/l (%)
PBS	20.5 ± 3.0	0.1 ± 0.1 (0.5 ± 0.3)	0.3 ± 0.2 (1.0 ± 0.7)	0.1 ± 0.06 (0.5 ± 0.3)	20.1 ± 2.9 (98.0 ± 1.1)
<i>S. chartarum</i>	99.6 ± 20.5	15.4 ± 0.3 (17.0 ± 4.9)	15.2 ± 4 (14.6 ± 2.7)	9.1 ± 1.2 (10.8 ± 1.9)	59.8 ± 18.1 (57.8 ± 5.6)
OVA/ <i>S. chartarum</i>	508.1 ± 64.5*	343.9 ± 49.3* (66.6 ± 3.4)	71.0 ± 17.8* (12.2 ± 1.9)	33.0 ± 3.6* (7.6 ± 0.8)	60.2 ± 6.9† (13.6 ± 2.4)
OVA	158.1 ± 23.5	116.5 ± 20.2 (72.5 ± 2.1)	2.8 ± 0.9 (1.8 ± 0.5)	19.2 ± 4.0 (11.5 ± 1.4)	19.6 ± 2.1 (14.2 ± 2.3)

Definition of abbreviations: OVA = ovalbumin; PBS = phosphate-buffered saline. Mice were sensitized/challenged and exposed intranasally according to the protocol shown in Figure 1. The groups are the same as in Figure 1. Bronchoalveolar lavage fluid was obtained from the mice 24 h after the last airway challenge as described in METHODS. The cells were differentiated in a blind fashion by counting at least 200 cells under light microscopy. The results are expressed as mean ± SEM. * Significantly different from the other groups. † Significantly different from the other groups, but not from *S. chartarum*-exposed-only group.

S. chartarum compared with mice exposed to *S. chartarum* only (Figure E1 of the online supplement). The lower CD4/CD8 ratio, indicative of an increase in CD8⁺ T cells, was at the same level as for the OVA-sensitized/challenged mice. However, taken together with the increased overall inflammation in mice sensitized/challenged to OVA and exposed to *S. chartarum*, and especially in relation to the significant increase in lymphocytes, the absolute amount of CD8⁺ lymphocytes was markedly increased in this group.

PAS staining of the sections showed an increase in mucus production in OVA-sensitized/challenged mice (Figures 2G and 2H). The amount of PAS-positive cells per 100 μm of the bronchial wall is shown in Figure 2J. There was no major difference in the numbers of mucin-producing cells between the allergic mice exposed to *S. chartarum* and the mice not exposed to *S. chartarum*.

Cytokine Levels in the Lungs Are Modulated Differently by Allergen and *Stachybotrys* Exposure

Proinflammatory cytokine mRNA expressions were markedly up-regulated in the OVA-sensitized/challenged mice exposed to *S. chartarum* spores (Figures 3A and 3B). IL-1β and TNF-α were induced in all treatment groups but were markedly elevated in the OVA-sensitized/challenged mice exposed to *S. chartarum* as compared with the *S. chartarum*-exposed or the OVA-sensitized/challenged mice. In the BALF, the TNF-α level was increased by up to 100-fold as compared with the OVA-sensitized/challenged mice and by almost 20-fold as compared with the *S. chartarum*-exposed mice (Table 2).

OVA-sensitized/challenged mice exposed to *S. chartarum* exhibited mixed Th1 and Th2 cytokine responses (Figures 3C–3E). The mRNA expression levels of IL-4, IL-5, and IL-13 were high in the OVA-sensitized/challenged mice, and the levels were slightly,

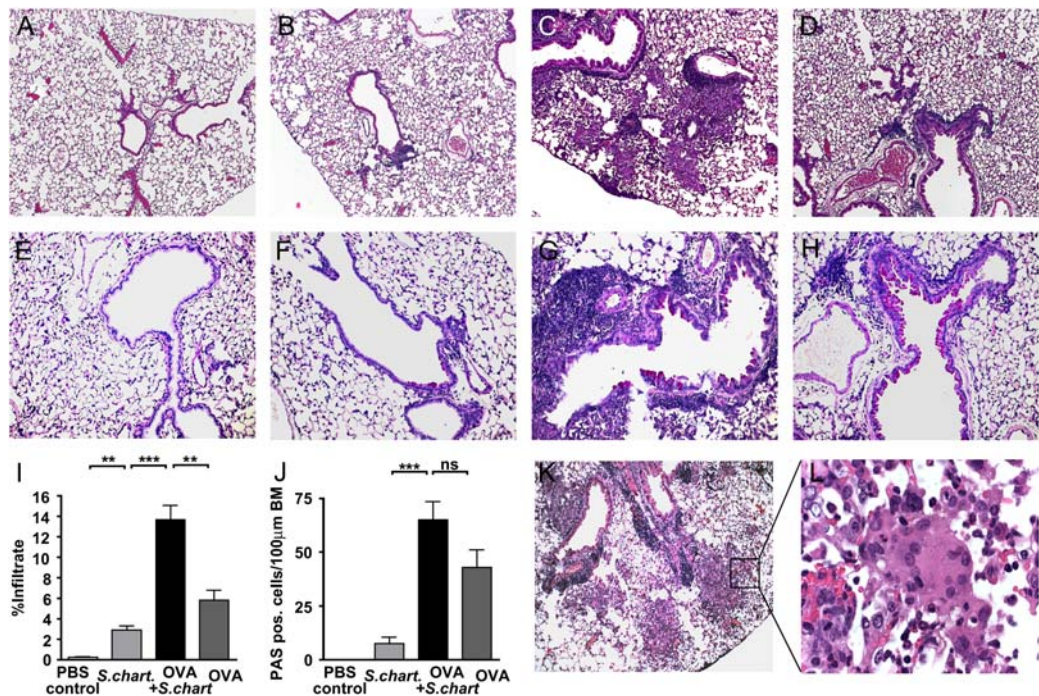


Figure 2. Histologic lung sections from treated mice. Representative hematoxylin and eosin (H&E)-stained lung sections (original magnification: ×10) are shown from a PBS control mouse (A), an *S. chartarum*-exposed mouse (B), an OVA-sensitized/challenged mouse exposed to *S. chartarum* spores (C), and an OVA-sensitized/challenged mouse (D). The sensitization and exposure protocol is described and shown in Figure 1. Periodic acid Schiff (PAS)-stained sections (original magnification: ×20) from the corresponding groups are shown below in E–H. The degree of inflammation in the H&E-stained lung sections was estimated by counting the number of squares showing inflammatory infiltrates per 100 squares from three different sites in the lung. Data are presented as percent infiltrate in I. The frequency of mucin-producing cells in the

basement membrane (BM) was counted, and the results are presented as PAS positively stained cells/100 μm of BM in J. Multiple granulomas (K) with giant cells (L; original magnification: ×100) can be seen in lung tissue from an OVA-sensitized/challenged mouse exposed to *S. chartarum* spores. Significant differences between the groups at **p < 0.01; ***p < 0.001. Note the inflammatory cell clusters in the OVA-sensitized/challenged and *S. chartarum*-exposed mice seen peribronchially, perivascularly, and in the alveoles.

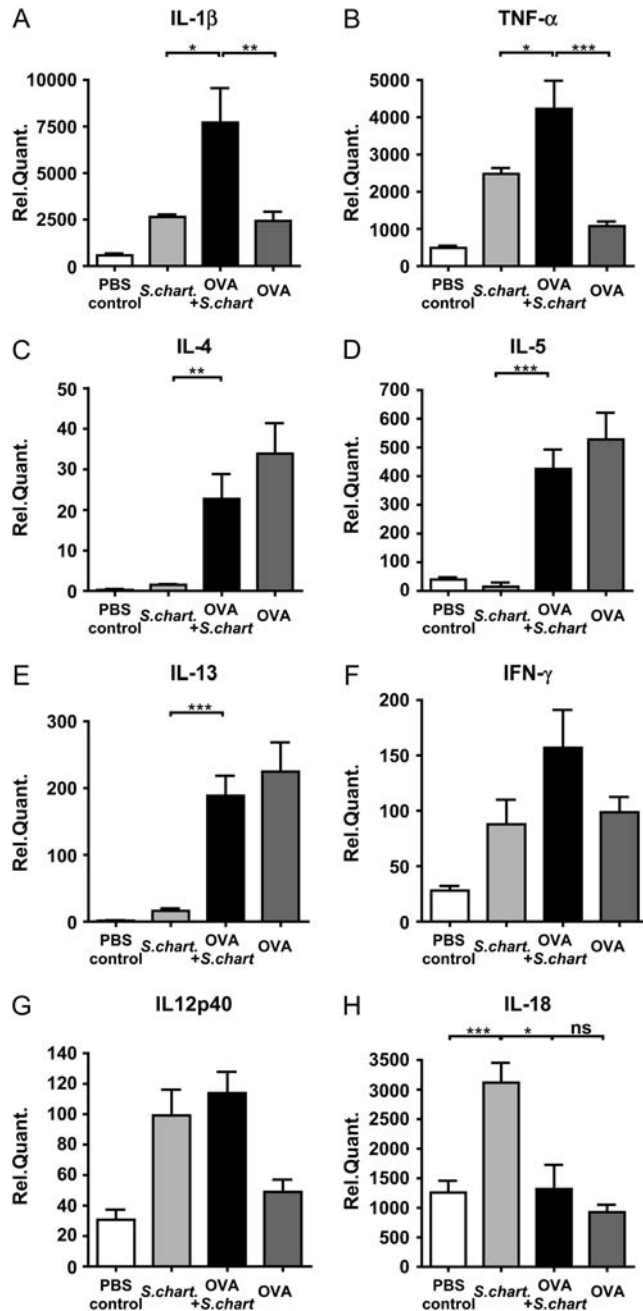


Figure 3. (A, B) Proinflammatory cytokine mRNA expressions are strongly induced in the lung of OVA-sensitized/challenged mice exposed to *S. chartarum* spores. (C–E) Th2 cytokine mRNA expression levels. (F–H) Th1 cytokine mRNA expression levels. The mice were sensitized/challenged and exposed to intranasal instillations as shown in Figure 1. RNA was extracted from the lungs, and the cytokine mRNA expressions were measured by real-time quantitative polymerase chain reaction (PCR) method. The results are expressed as relative units of PCR product formed. Results are expressed as mean $\pm$ SEM for each group. Significant differences between the groups at * p < 0.05; ** p < 0.01; *** p < 0.001.

although not significantly, reduced when the mice were also exposed to *S. chartarum* spores. Mice exposed to *S. chartarum* showed no induction of Th2 cytokines because the levels in these mice were similar to the PBS control mice. Similar changes of the protein level of IL-13 were seen in the BALF (Table 2).

TABLE 2. TUMOR NECROSIS FACTOR- α AND INTERLEUKIN-13 LEVELS IN BRONCHOALVEOLAR LAVAGE FLUID

Group	TNF- α (pg/ml)	MIP-1 α (pg/ml)	IL-13 (pg/ml)
PBS	0.5 $\pm$ 0.5	0.0 $\pm$ 0	2.8 $\pm$ 0.9
<i>S. chartarum</i>	9 $\pm$ 9.0	1.0 $\pm$ 1.0	4.8 $\pm$ 1.2
OVA/ <i>S. chartarum</i>	171.1 $\pm$ 55.8*	6.6 $\pm$ 3.8	69.1 $\pm$ 17.0†
OVA	1.8 $\pm$ 1.8	1.5 $\pm$ 0.8	72.9 $\pm$ 20.1

Definition of abbreviations: IL = interleukin; MIP = macrophage inflammatory protein; OVA = ovalbumin; PBS = phosphate-buffered saline; TNF = tumor necrosis factor.

Mice were sensitized/challenged and given intranasal instillations as shown in Figure 1. One day after the last airway challenge, the mice were killed, and the bronchoalveolar lavage fluid was collected. The cytokines were measured by ELISA. Results are expressed as mean $\pm$ SEM for each group.

* Significantly different from the other groups.

† Significantly different from the other groups but not from mice in the OVA-sensitized/challenged-only group.

The Th1-type cytokines, IFN- γ , and IL-12p40 were up-regulated in all exposure groups and showed a slight tendency to increase in the OVA-sensitized/challenged mice exposed to *S. chartarum* spores, as compared with OVA-sensitized/challenged or *S. chartarum*-exposed mice (Figures 3F and 3G). IL-18 expression was increased only in the mice exposed to *S. chartarum* spores, and the expression was down-regulated in the OVA-sensitized/challenged mice exposed to *S. chartarum* spores to the same level as found in control mice (Figure 3H).

CCL2 and CCL3 Chemokines Are Markedly Induced in Allergic Mice Exposed to *S. chartarum*

CCL2/MCP-1 and CCL3/MIP-1 α attract inflammatory cells in general, whereas MIP-2 is a chemokine that specifically attracts neutrophils in mice. CCL2, CCL3, and MIP-2 were induced in all treatment groups but were markedly induced in OVA-sensitized/challenged mice also exposed to *S. chartarum* (Figure 4). The levels of CCL3/MIP-1 α protein were similarly increased in the BALF from OVA-sensitized/challenged mice exposed to *S. chartarum* (Table 2). On the other hand, the expression of CCL11/eotaxin-1, which is involved in the recruitment of eosinophils, was not significantly modulated by the *Stachybotrys* exposure (data not shown). The chemokine receptors CCR5, CCR3, and CXCR2, selected for some of the chemokine ligands mentioned previously, were induced in all treatment groups (Figures 4D–4E). The expressions were strongly induced in the OVA-sensitized/challenged mice exposed to *S. chartarum* spores.

AHR Is Reduced by *S. chartarum* Exposure in Allergic Mice

As shown in our previous study and further confirmed here, exposure to *S. chartarum* did not induce any significant increase in the AHR when compared with the PBS-treated control mice. AHR was increased in the OVA-sensitized/challenged mice as expected (Figure 5), but when the OVA-sensitized mice were also exposed to *S. chartarum* spores, the AHR decreased to the same level seen in mice exposed to *S. chartarum* alone.

Antibody Levels in Serum

Total IgE concentrations and OVA-specific IgE, IgG2a, and IgG1 levels in serum were high but were at the same level in the OVA-sensitized/challenged mice and the OVA-sensitized/challenged mice exposed to *S. chartarum* spores (data not shown). No total IgE- or OVA-specific antibodies were detected in mice exposed to *S. chartarum* only. No significant increases in *S. chartarum*-specific antibodies were detected in any of the groups (data not shown).

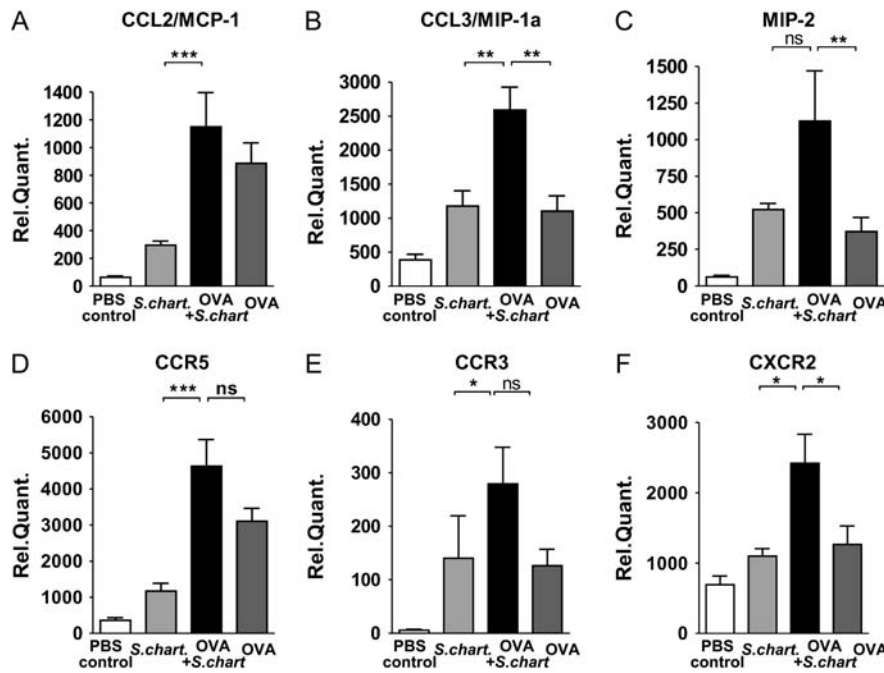


Figure 4. Inflammatory chemokines (A–C) and chemokine receptors (D–F) are markedly induced in OVA-sensitized/challenged mice exposed to *S. chartarum* spores. Mice were sensitized/challenged and given intranasal instillations according to the protocol shown in Figure 1. Total RNA was extracted, and the expression level was measured as described in Figure 3. Significant differences between the groups at * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Expression of TNF- α and IL-1 β Is Markedly Enhanced in Splenocytes from OVA-sensitized Mice by *S. chartarum* Stimulation

To explore cellular mechanisms of the *S. chartarum*-induced allergic lung inflammation, splenocytes from OVA-sensitized and naive OVA-TCR transgenic mice were stimulated *in vitro* by *S. chartarum*. Expression of TNF- α and IL-1 β was markedly enhanced by *S. chartarum* in splenocytes from OVA-sensitized

mice but not in naive mice (Figure 6A). OVA stimulation elicited significant induction of IL-13 mRNA in OVA-sensitized mice, confirming Th2 environment of the splenocytes (Figure 6B).

DISCUSSION

Exposure to damp building molds has been associated with many adverse respiratory health effects, such as cold- and flulike symptoms and exacerbations of asthma (19). The causal link between the exposure and symptoms has proved elusive to identify. Some data suggest that atopic individuals may be more sensitive to exposure to molds exhibiting increased respiratory symptoms as compared with nonatopic healthy individuals (20). The effect of a damp building mold may, therefore, depend on the atopic status of the individual exposed.

Previously, we have shown that exposure to *S. chartarum* induced an influx of neutrophilic and lymphocytic cells in the mouse lung; this is paralleled by the appearance of a proinflammatory cytokine profile (16). This study was designed to determine whether the pulmonary inflammatory and physiologic response against a damp building mold, *S. chartarum*, is different in allergic mice. We used a model of established OVA sensitization/challenge that leads to a severe pulmonary inflammation with increased AHR (17) in conjunction with intranasal *S. chartarum* exposure. We were able to demonstrate a mixture of a proinflammatory response and an eosinophilic, allergy-type of response. Moreover, pulmonary inflammation was enhanced by *S. chartarum* exposure.

The inflammation in the lungs of the mice that had been OVA sensitized/challenged and exposed to *S. chartarum* spores was more severe than in the OVA-sensitized/challenged mice or mice exposed to *S. chartarum* alone. Data from the BALF samples and histologic lung sections indicated that not only did the total number of cells strongly increase, but also the cell population changed. It shifted from an eosinophilic infiltrate in the OVA-sensitized/challenged mice toward a mixture of eosinophils, neutrophils, and lymphocytes in the OVA-sensitized/challenged exposed to *S. chartarum* spores. In addition,

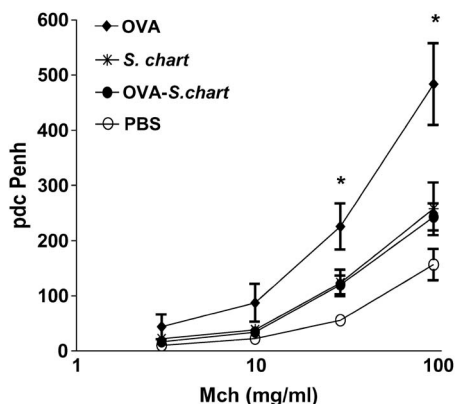


Figure 5. Intranasal instillations of *S. chartarum* spores decreases airway hyperresponsiveness in OVA-sensitized/challenged mice. Mice were sensitized/challenged and exposed to intranasal instillations as shown in Figure 1 and as described in METHODS. Airway responsiveness to increasing concentrations of nebulized MCh (0–100 mg/ml) was assessed 24 h after the last airway challenge by whole-body plethysmography. After each nebulization, recordings were taken for 5 min. The enhanced pause (Penh) values measured during each sequence were averaged and expressed for each MCh concentration as the percentage of baseline Penh values after PBS exposure (% baseline Penh, pdc). Results are expressed as the mean pdc Penh $\pm$ SEM for each group. * Significant differences between the groups.

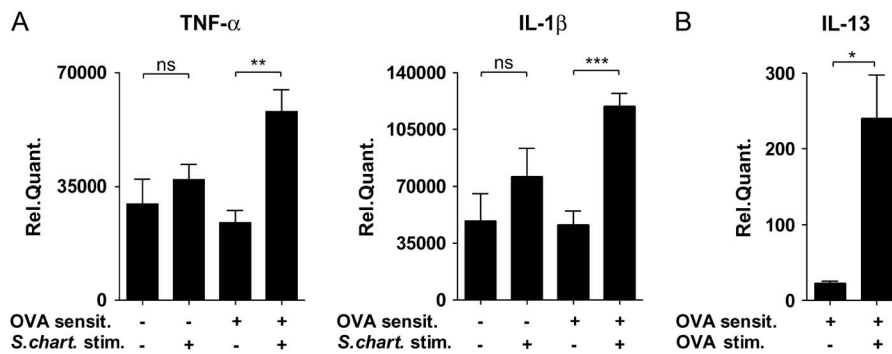


Figure 6. (A) Proinflammatory cytokine mRNA expression levels in splenocytes from naive or OVA-sensitized mice stimulated with *S. chartarum*. The splenocytes from naive or OVA sensitized OVA-TCR transgenic mice were exposed to *Stachybotrys* spores. RNA was isolated, and mRNA expression levels were measured as described in METHODS. (B) The Th2 cytokine IL-13 is clearly induced in OVA-stimulated splenocytes isolated from OVA-sensitized OVA-TCR transgenic mice. Results are expressed as mean $\pm$ SEM for each group. Significant differences between the groups at * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

multinucleated giant cells and epithelioid granulomas appeared in the lung sections. This inflammation resembles allergic alveolitis or hypersensitivity pneumonitis, a complex disorder of the lungs caused by repeated exposure to a variety of inhaled antigens. Hypersensitivity pneumonitis is characterized by a Th1 type of response with the induction of proinflammatory cytokines, influx of CD8⁺ lymphocytes, and granuloma formation (21). Multinucleated giant cells are known to produce TNF- α , and granulomatous inflammations are associated with a Th1 type of response with the production of TNF- α and IFN- γ (22, 23).

We have recently shown that repeated intranasal exposure to *S. chartarum* spores induces the expression of proinflammatory and Th1 cytokines in murine lungs. In the model of OVA-sensitization-induced allergic asthma, the expression of Th2 cytokines in the lungs predominate (18). In the present study, we examined the effect of *Stachybotrys* exposure to the allergic mice and observed that the proinflammatory cytokines IL-1 β and TNF- α were markedly increased, whereas the levels of the Th1/Th2 cytokines remained unchanged. IL-18, which was originally called IFN- γ inducible factor, acts in conjunction with IL-12 to stimulate the production of Th1-type cytokines and especially IFN- γ (24, 25). In our study, the IL-18 levels were induced only in the mice exposed to *S. chartarum*, whereas the levels were suppressed, probably by the Th2 response, when the mice were sensitized and challenged with OVA. Taken together, our results show that exposure to *S. chartarum* in combination with OVA sensitization and challenge induces high levels of proinflammatory cytokines.

The early signals in innate immune responses, such as the secretion of proinflammatory cytokines, are the main inducers for the release of chemokines that recruit inflammatory cells (26). The expression of CCL3/MIP-1 α mRNA was most intensely induced in the OVA-sensitized/challenged mice exposed to *S. chartarum* spores. CCL3 acts as a chemoattractant through the chemokine receptors CCR1 and CCR5, which are expressed on inflammatory and antigen-presenting cells. Elevated levels of chemokine receptor CCR5 often accompany a Th1 type of inflammatory response (27), and the levels of CCR5 were also clearly induced in the OVA-sensitized/challenged exposed to *S. chartarum* spores. The CCR3 chemokine receptor, which is typically expressed on eosinophils and CXCR2 receptor found on neutrophils, were also upregulated in this group, which may partly explain the influx seen in the BALF (28). Taken together, our results suggest that certain inflammatory chemokines and chemokine receptors orchestrating the lung inflammation are markedly induced in the OVA-sensitized/challenged mice that have been exposed to *S. chartarum* spores. The triggering factor

for this inflammatory cascade in these mice may be the intense induction of TNF- α found here at the mRNA and protein levels.

The mechanisms involved in the induction and development of AHR are not fully understood, but airway inflammation and remodeling have been suggested as causes of AHR (29). Studies have shown that the eosinophilic inflammation in the lungs, together with increased IL-5 levels, is closely, but not exclusively, associated with AHR in models of allergic asthma (2, 17, 18, 30). In this study, we saw a decreased AHR response even though there was intense pulmonary eosinophilic infiltration. Microbes or microbial products have been shown to down-regulate the AHR of allergic mice in recent studies (31–33). In these cases, however, bacteria-derived components reduced the eosinophilic inflammation and suppressed the IgE production, which was not the case in our study. It is possible that the effect on AHR seen here was attributable to changes in cytokine expression, such as the slight decrease in IL-13 and to some extent other Th2 cytokines (34). It remains to be seen whether there are some uncharacterized counter effects on the smooth muscle mediated by the intense TNF- α response and neutrophilic influx.

A hypothesis has been presented suggesting that Th1 cells may precede and favor the recruitment of Th2 cells in the lungs in allergic asthma. Chaplin and colleagues (35) demonstrated a mixed Th1 and Th2 CD4⁺ T-cell response in a model of eosinophil-predominant airway inflammation in mice. They showed by adoptive transfer of allergen-specific Th1 and Th2 cells that Th2 cells on their own were not as potent at initiating a tissue inflammatory response as the combination of Th1 and Th2 cells (36). A strong local proinflammatory stimulus, such as treatment with TNF- α , endotoxin, or infection with respiratory tract viruses, has been shown to enhance the recruitment of Th2 lymphocytes to the sites of antigen challenge (37, 38). Because our intranasal instillation of *S. chartarum* spores induced an influx of inflammatory cells resembling more the Th1 type and the OVA-allergic model of a Th2 type, we may also here be witnessing an enhanced recruitment of Th2 lymphocytes due to the combination of two types of responses.

The mechanism behind the *S. chartarum*-induced severe lung inflammation seen here is unclear. Because robust lung inflammation was seen only in mice that were allergen sensitized in parallel with exposure to *S. chartarum*, we hypothesized that the Th2 milieu might play an important role in modulating the response to *S. chartarum*. In an attempt to elucidate the mechanism behind the marked increase in proinflammatory cytokines in this study, we isolated splenocytes from naive and OVA-sensitized OVA-TCR transgenic mice and exposed them to *Stachybotrys* spores *in vitro*. Our results clearly demonstrate that

allergen sensitization is a prerequisite for vigorous expression of TNF- α and IL-1 β in response to *S. chartarum* stimulation, which may critically contribute to the development and maintenance of the lung inflammation.

In conclusion, we have demonstrated that the effects of exposure of the damp building mold *S. chartarum* strongly depend on the allergic status of the mice. We were able to demonstrate a synergistic effect between the allergen sensitization and mold exposure, resulting in enhanced and qualitatively different granulomatous pulmonary inflammation as reflected by increases in proinflammatory cytokines and chemokines and the cell infiltration. These changes were accompanied by decreased hyperresponsiveness, implying that although *Stachybotrys* exposure can induce various and often unpredictable immunobiologic effects on the respiratory system, triggering of an asthmatic response may not be a major factor.

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

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