Chronic intranasal administration of mold spores or extracts to unsensitized mice leads to lung allergic inflammation, hyper-reactivity and remodeling.

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Summary

Allergic asthma is a serious multifaceted disease characterized by eosinophil-rich airway inflammation, airway hyper-reactivity (AHR) and airway wall modifications called remodeling. We previously demonstrated that the spores of two allergenic molds *Alternaria alternata* and *Cladosporium herbarum*, were potent inducers of IgE production. Moreover mice sensitized by two intraperitoneal (i.p.) injections before intranasal (i.n.) challenge with *A.alternata* or *C.herbarum* spores developed an allergic lung inflammation and hyperreactivity. Here we report on the effect of a chronic i.n. administration of *C.herbarum* spores or *A.alternata* extracts to unsensitized BALB/c mice. Our results demonstrate that this chronic treatment leads to an increase of total serum IgE and the appearance of specific IgE and IgG1. Total cell number in bronchoalveolar lavages (BALF) was highly increased as compared to PBS treated mice due to an accumulation of macrophages, neutrophils, lymphocytes and eosinophils. AHR appeared after 3 weeks (extract) and 7 weeks (spores) and was maintained during the whole treatment. Increased IL-13 mRNA expression in the lungs and Th2 cytokines (interleukin-4, -5, -6 and -13) and transforming growth factor-β secretion in BALF was also observed. Lung hydroxyproline and fibronectin contents indicated increased fibrosis in mold allergen treated mice. These observations were confirmed by histological analysis demonstrating an airway wall remodeling and a strong mucus production. These observations show that this model, using chronic i.n. administration of relevant particulate allergens, is an interesting tool to study the mechanisms leading to allergic pulmonary diseases and lung remodeling.

Introduction

Allergic asthma is a multifactorial chronic disease involving both genetic and environmental factors and characterized by a dysregulation of immunity. This dysregulation leads to a strongly polarized Th2 immune response and a chronic inflammation in the airways in response towards innocuous antigens as for instance airborne allergens. [1] In asthmatic patients, the penetration of allergens into the lungs leads to an airway inflammation consisting of a peri-bronchial infiltration of $CD4^+$ T cells, macrophages, eosinophils and neutrophils and the presence of these cells in bronchoalveolar lavages (BALF). Asthmatic patients also present a goblet cell metaplasia/hyperplasia and characteristic modifications of the airway wall including epithelial hyperplasia, thickening of the basement membrane, subepithelial fibrosis, increased airway smooth muscle mass and finally an airway hyperreactivity (AHR) to specific and non specific stimuli. [2, 3]

So far, most experimental models of asthma have been developed in rodents and have used chicken egg ovalbumin (OVA) as a surrogate allergen. Although these OVA models have been valuable tools for the study of inflammatory mechanisms of asthma, OVA bears little relationship with aeroallergens present in daily environment and the use of common aeroallergens involved in human asthma may be more relevant to study the pathophysiology of this chronic disease. Indeed the administration of OVA through the airway is incapable of inducing a broad spectrum of lung allergic changes and on the contrary induces a relative persistent tolerogenic state. [4,5] However, OVA is readily capable of inducing goblet cell metaplasia, mucus oversecretion, airway eosinophilia, peribronchovascular inflammation and airway hyperresponsiveness if given systemically in a series of priming doses, typically with aluminum-based adjuvants

before pulmonary challenge. [6,7] These models based upon sensitization by systemic administration of antigen (such as OVA) and subsequent short term challenge by inhalation are experimentally very convenient and have been widely used. However these short term exposure models are quite unlike the recurrent long-term exposure to allergens experienced by humans. Therefore chronic inhalational challenge models of asthma, in previously sensitized mice, using controlled exposure to nebulized OVA have also been developed [8, 9] allowing to study the chronic inflammatory and epithelial changes that typify asthma.

Recently we described a new acute mouse model of lung allergy induced by the spores of two well-recognized human allergenic molds, *Alternaria alternata* and *Cladosporium herbarum*. [10] In contrast to Aspergillus which is an opportunistic pathogen causing allergic and invasive diseases, spores from Alternaria and Cladosporium do not colonize the lungs and are rapidly cleared. Spores from these two molds are important causes of both allergic rhinitis and asthma and exposure to airborne spores of *A.alternata* might trigger severe asthma and represents a risk factor for respiratory failure. [11] We previously demonstrated that mice systemically sensitized with *A.alternata* and *C.herbarum* spores and then intranasally (i.n.) challenged developed a typical allergic airway inflammation and AHR. [10]

In this study we have analyzed the effect of chronic i.n. instillations of mold spores and extracts in naive mice and we demonstrate that this treatment induces a typical Th2 immune response characterized by a polyclonal IgE and a specific IgE-IgG1 synthesis, a lung inflammation containing numerous eosinophils, the development of an AHR and an important remodeling of the airway wall including epithelial hypertrophy, goblet cell hyperplasia/metaplasia and subepithelial fibrosis.

Materials and methods

Mold cultures, spore production and mold extract preparation

Alternaria alternata (strain18586) was obtained from the BCCM™/IHEM (Institute of Public Health, Brussels, Belgium). *Cladosporium herbarum* (strain 19275) was obtained from the BCCM™/MUCL (Université Catholique de Louvain, Louvain, Belgium). These molds were cultured at 27°C on potato dextrose agar (Difco, detroit, USA) plates for one week before gently harvesting the spores with a cell scraper. Spores were diluted in PBS and counted with a hemocytometer.

Mold extracts were prepared as previously described [12] with slight modifications. Mold cultures were grown for 3 weeks at 27°C in flasks containing 250 ml of Czapek's medium. Mold pellicles were harvested and homogenized in 0.4% NH_4HCO_3 + polyvinyl polypyrrolidone (Sigma) using an Ultra-Thurax. The homogenates were then agitated for 3 hours at 4°C. Extracts were centrifuged twice for 30 min. at 20,000g, dialyzed against PBS and stored at –20°C.

Animals and immunizations.

Female BALB/c mice were obtained from the Elevage Janvier (Le Genest Saint Isle, France) and maintained under standard laboratory conditions. To induce the allergic lung inflammation, mice were instilled intranasally with 2.10^5 spores or 5 µg of extract (in 100 µl of PBS), twice a week, every Monday and Friday for ten to twelve weeks. Similar experiments were also performed with *C.herbarum* extracts and *A.alternata* spores (not shown). Results obtained after a chronic instillation of *C.herbarum* extract were very similar to those obtained with the *A.alternata* extract. Chronic instillation of 2.10^5 *A.alternata* spores induced a cachexia, probably caused by a high production of

TNF-α in response to this quantity of spores and therefore experiments were stopped after 5 to 7 weeks.

Control mice were instilled with PBS. Mice were lightly anesthetized with isofluran (Isoflo, Abbott laboratories, North Chicago, USA). When the mice were unresponsive but breathing comfortably, 100 µl of the spore or extract solution was directly applied on the nostrils. The animals were allowed to slowly inhale the liquid and then to recover in a supine position.

For intra-peritoneal (i.p.) immunizations mice were injected with 50µg of Keyhole Limpet Hemocyanin (KLH, Pierce) in a final volume of 300 µl, emulsified in Complete Freund Adjuvant (CFA, Pierce) and boosted two weeks later with 50 µg of KLH emulsified in incomplete Freund Adjuvant (IFA, Pierce). Similarly mice were immunized twice with KLH emulsified in Aluminum hydroxyde (Alum) (Imject Alum, Pierce).

In all experiments, data shown are representative of at least two independent experiments (except once for Figure 3.) Results were analysed using the paired student *t* test. Significant *p* values are indicated.

Antibody detection.

Mice were bled in the retro-orbital plexus 24 hours after the last i.n. instillation and antibodies in the sera were analyzed by ELISA. Total serum IgE levels were determined using a sandwich ELISA. Plates were coated with a rat anti-mouse IgE mAb (LO-ME-2, IMEX, UCL, Brussels, Belgium) and saturated with proteins from skimmed milk. Serial twofold dilutions of serum or purified monoclonal mouse IgE (LB-4, IMEX) were applied for 2 h. Peroxidase labeled rat anti-mouse IgE (LO-ME-3) was then added.

Finally plates were washed and developed by the addition of 100 µl of TMB (Immunopure TMB substrate kit, Pierce Biotechnology, Rockford, USA). The reaction was stopped with 50 µl of 2N H_2SO_4 and O.D. was read at 450 nm with an automatic Multiskan Reader MCC/340 (Titertek, Huntsville, USA). Serum titer was converted to IgE concentration by comparison with a purified LB-4 standard. Similar immunoglobulin concentrations for serum were calculated when serum and standard were titrated at any point of the linear part of the titration curve.

Mold specific IgE and IgG1 levels were determined using an indirect ELISA. Plates were coated with mold extracts and saturated. Serial twofold dilutions of serum were applied for 16 h. Peroxidase labeled rat anti-mouse IgE (LO-ME-3) or anti-mouse IgG1 (LO-MG1-13) were then added during 3 h. Finally plates were washed and developed by the addition of 100 µl of TMB.

In vitro spleen cell restimulation

Spleens were removed aseptically one week after the second KLH immunization. Spleen cells ($5x10^5$ cells in 200µl) were cultured in triplicate in 96 well round-bottom microwell plates in RPMI 1640 medium supplemented with gentamycin, 2mM L-glutamine, 5.10^{-5} M 2-mercaptoethanol, 10% FCS with or without KLH (10µg/ml) or Pokeweed Mitogen (PWM, 4µg/ml, Sigma). Cultures were maintained in 5% CO_2 at 37°C for three days and then supernatants were collected and analysed by ELISA for the presence of IFN-γ, IL-4 and IL-13.

Bronchoalveolar lavages.

Mice were euthanized by cervical dislocation 24 hours after the last i.n. instillation. The trachea was exposed and incised. A needle (1.2x40 mm) was inserted into the trachea and BAL fluid was harvested by rinsing the lungs twice with 1 ml of PBS. Total cell counts were determined with a hemacytometer. Differential cell counts were obtained by examining at least 500 cells on cytospin slides stained with Diff-Quick (Dade Behring, Deerfield, USA).

Measurement of airway reactivity

AHR was measured within 24h after the last instillation, in response to methacholine inhalation using a whole-body plethysmography system (EMKA, Paris, France). Mice were placed in the plethysmograph box and allowed to acclimatize for at least 5 min before analysis. Control measurements were obtained over a period of 5 min directly after a 3 min nebulization of PBS generated by an ultrasonic nebulizer (LS Syst'AM, France). Afterwards, increasing concentrations of methacholine (12.5, 25 and 50 mg/ml) in PBS were nebulized into the plethysmograph box for 3 min. Immediately after each nebulization the enhanced pause (P_{enh}), a dimensionless index that reflects changes in the amplitude of the pressure waveform and expiratory time, were recorded and averaged for 5 min. P_{enh} measurements have been validated by Hamelmann et al. [13] in regard to identification of AHR since the heightened increase in P_{enh} with methacholine challenge in OVA-sensitized/challenged mice was accompanied by a parallel enhancement of lung airway resistance (R_L) responses to the methacholine with a high correlation between P_{enh} and R_L.

Total RNA extraction and IL-13 mRNA quantification

Total RNA was extracted from perfused lungs with Trizol (Invitrogen), according to the manufacturer's instructions. Residual DNA contamination was removed by treatment of the samples with DNA-free kit (Ambion, Austin, USA). One µg RNA was reverse transcribed using SuperScript III Reverse Transcriptase (Invitrogen) with 350 pmole random hexamers (Eurogentec, Seraing, Belgium) in a final volume of 25 µl. Resulting cDNA was then diluted 10x and used as template in subsequent polymerase chain reaction (PCR).

Specific primer sets were as follows (Invitrogen): IL-13 sense AGACCAGACTCCCCTGTGCA, IL-13 antisense TGGGTCCTGTAGATGGCATTG, IL-13 probe (FAM-TAMRA) CGGGTTCTGTGTAGCCCTGGATTCC, ß-actin sense AGAGGGAAATCGTGCGTGAC, ß-actin antisense CAATAGTGATGACCTGGCCGT. For IL-13 and ß-actin mRNA quantification, standards and samples (2,5 µl) were amplified with 300 nM of each primer and probe using respectively Taqman PCR Master Mix (Applied Biosystems, Foster City, USA) and SYBR Green PCR Master Mix (Applied Biosystems) in a total volume of 20 µl. PCR was performed on an ABI Prism 7000 Sequence Detector (Applied Biosystems) according to the following program : 2 min 50°C, 10 min 95°C, (15 s 95°C, 1 min 60°C) x40. Serial dilutions of a positive control sample of cDNA were used as standards for quantification. Results were calculated as a ratio of IL-13 expression to the expression of the reference gene, ß-actin.

Cytokine detection

Cytokines were measured in BALF obtained 24 hours after the last i.n. inoculation. The levels of IL-4, IL-5, IL-6, IL-10, IL-13, IFN-γ and total TGF-β were quantified by ELISA (sensitivity were 2 pg/ml, 7 pg/ml, 1.8 pg/ml, 4 pg/ml, 1.5 pg/ml, 2pg/ml and

4.6pg/ml respectively) using Quantikine® kits according to the instructions provided by the manufacturers (R&D systems, Minneapolis, USA).

Quantification of fibrosis markers

Collagen deposition was estimated by measuring the hydroxyproline content of whole lung and soluble collagen in lung homogenates. For hydroxyproline assays, the lung was excised, homogenized, and hydrolyzed in 6N HCl overnight at 110°C. Hydroxyproline was assessed by high-performance liquid chromatography analysis [14] and data were expressed as micrograms of hydroxyproline per lung. Soluble collagen levels were estimated by Sircol collagen assay following the manufacturer's protocols (Biocolor, Westbury, USA). Fibronectin was measured in BALF by ELISA as previously detailed [15].

Histological analysis.

Tissues for histopathological examination were collected 24 hours after the last i.n. instillation. Mice were euthanized by cervical dislocation, the trachea was canulated and the lungs were inflated with 4% buffered formalin. After fixation overnight, the lungs were embedded in paraffin. Tissues were sliced and 5 µm sections were stained with Hematoxylin-Eosin-Safran (HES) for light microscopy examination of the lung inflammation, with Periodic Acid Shiff (PAS) for the detection of mucus producing cells and Masson trichrome for the detection of collagen.

Results

Chronic i.n. instillation of A.alternata extract or C.herbarum spores into naïve BALB/c mice induces a polyclonal IgE production and a secretion of specific IgG1 and IgE antibodies

We previously demonstrated that the spores of *A.alternata* and *C.herbarum* are potent inducers of polyclonal IgE and specific IgG1 antibodies synthesis when injected twice i.v. to BALB/c mice [10]. In order to analyse the ability of mold spores and extracts to trigger a systemic Th2 response when introduced chronically into the airways, naive BALB/c mice were instilled i.n. twice a week with *A.alternata* extracts (5µg) or *C.herbarum* spores (2.10^5 spores) and ten weeks later antibody synthesis and IgE serum levels were measured. As shown in Figure 1, at the end of this treatment, total serum IgE levels were highly increased in both groups as compared to the PBS-treated control group and there was no significant difference between IgE levels of mice instilled with the spores or the extract. The chronic i.n. administration of mold spores or extracts also led to the production of specific IgE and IgG1 antibodies in the serum (Figure 2) although these synthesis were lower in mice instilled with spores than with extract. PBS-instilled mice did not produce any specific IgE or IgG1 antibodies. We were unable to detect any secretion of specific IgG2a antibodies in mold spore or extract-treated mice (not shown). These results clearly demonstrate that these antigens are able to induce systemic type 2 response when introduced chronically into the airways. In order to analyse the consequences of this systemic type 2 activation on specific antigenic responses, mice were chronically instilled with *A.alternata* extract or PBS during three weeks. Then mice were immunized i.p. with KLH emulsified in CFA or Alum and boosted with KLH in IFA or Alum respectively. One week after the boost

spleens were removed and the cells were restimulated *in vitro* with medium, KLH or PWM. Supernatants were collected and analysed for the presence of the prototypal Th1 cytokine IFN-γ and the Th2 cytokines IL-4 and IL-13. As shown in Figure 3, spleen cells from mice immunized with KLH emulsified in CFA/IFA or with Alum and restimulated with PWM produced high levels of IFN-γ, IL-4 and IL-13. When restimulated with KLH, spleen cells from mice immunized with KLH emulsified in CFA/IFA secreted more IFN-γ while cells from mice immunized with KLH in Alum produced more IL-4 and IL-13. However there was no difference in the secretion of these cytokines between mice instilled with the mold extract and control PBS mice, indicating that the systemic Th2 activation induced by chronic mold exposure did not influence the T cell response induced by i.p. injection of an unrelated antigen.

Chronic i.n. instillation of A.alternata extracts or C.herbarum spores induces the appearance of inflammatory cells in BALF

BALB/c mice sensitized by two i.p. injections of *A.alternata* or *C.herbarum* spores and then challenged i.n. develop a strong allergic inflammation into the airways consisting of an accumulation of macrophages, neutrophils, eosinophils and lymphocytes around the bronchiols and an accumulation of these cells in their BALF [10]. To investigate the type of inflammation induced during a chronic exposure to mold allergens, BAL cells were examined in naive BALB/c mice chronically instilled i.n. for 10 weeks with PBS, *A.alternata* extracts or *C.herbarum* spores. The presence of inflammatory/immune cells was detected in BAL after 3 (extract) to 5 (spores) weeks of treatment (not shown). After ten weeks of treatment, the total number of cells obtained in the BAL of mold spores or extract treated mice were highly increased as compared to the number of cells

In BALF from the PBS treated group (Figure 4A). Analysis of the different cell subpopulations showed that i.n. spores or extracts instillations led to the recruitment of a high number of macrophages, neutrophils, lymphocytes and eosinophils (Figure 4B). This increase in the number of eosinophils in the airways has been considered as a hallmark of allergic asthma [3].

Chronic i.n. instillation of A.alternata extracts or C.herbarum spores leads to a sustained airway hyperreactivity.

Whole body plethysmography was used to assess the development of AHR. Measured P_{enh} values from BALB/c mice chronically instilled i.n. during 10 weeks with *A.alternata* extracts or *C.herbarum* spores were compared to those from PBS treated mice. In PBS treated mice, no AHR was observed in the course of the experiment. Mice chronically instilled with *A.alternata* extracts did not show any AHR during the first two weeks of treatment. On week 3 AHR appeared and remained stable during the whole treatment (Figure 5). In contrast P_{enh} values of mice instilled with *C.herbarum* spores were only slightly increased at week 3 and 5 but strong AHR was obtained at week 7 and 9. Two weeks after the end of the instillation AHR of *A.alternata* extracts or *C.herbarum* spores treated groups was not modified as compared to AHR observed after 9 weeks of i.n. instillation. Six and ten weeks after the end of treatment, AHR decreased but was still above baseline levels observed in the control PBS-treated group.

Chronic i.n. instillation of A.alternata extracts or C.herbarum spores increases Th2 cytokine lung production

IL-4, -5 and -13 are Th2 cytokines playing key regulatory roles during allergic airway inflammation [16-19]. Expression of IL-13 mRNA was measured by quantitative RT-

PCR in the lungs of extract treated mice as compared to PBS treated mice. Figure 6A shows that IL-13 mRNA was already strongly over-expressed in the lungs of mold extract treated mice after 3 weeks and remained constant during the whole experiment while IL-13 expression was not increased in PBS control mice as compared to untreated mice. The presence of IL-13 in BALF was confirmed by ELISA after 10 weeks of treatment. As shown in Figure 6B a low concentration of IL-13 was found in BALF from control mice while IL-13 was significantly increased in BALF from mice chronically instilled with either *A.alternata* extract or *C.herbarum* spores. The concentrations of IL-4, IL-5, IL-6 and IL-10 were also measured by ELISA in the BALF of *A.alternata* extract treated mice. A significantly higher concentration of IL-4, IL-5 and IL-6 was observed in BALF from extract treated mice as compared to PBS treated mice while IL-10 concentrations showed no significant variation. Interestingly BALF levels of the pro-fibrotic mediator TGF-β were also increased in mice treated with *A.alternata* extracts (Figure 7). Finally, IFN-γ was below detection level in these BALF (not shown). These data clearly indicate that the mold extract and spores are strong and specific inducers of Th2 immunity.

Chronic i.n. instillation of A.alternata extracts or C.herbarum spores induce an airway wall remodeling.

Chronic human asthma is characterized by a chronic airway inflammation but also by modifications of the airway wall, called remodeling, such as subepithelial fibrosis, mucus production and epithelial proliferation. The amplitude of the pulmonary fibrosis induced by the chronic i.n. instillation of *A.alternata* extracts was determined after 10 weeks of treatment by measuring lung hydroxyproline, soluble collagen and fibronectin,

as well as by histology. As compared to PBS treated animals, mice exposed to *A.alternata* extracts presented an increase of hydroxyproline and soluble collagen and a high concentration of fibronectin in the BALF (Figure 8). Histological examination of *A.alternata* extract and *C.herbarum* spores treated mice confirmed the biochemical evidence of a remodeling. Masson's trichrome staining of lung sections showed a pronounced thickening of the airway epithelium of mold extract or spore treated mice as compared to PBS treated mice and more importantly a strong accumulation of collagen beneath the airway epithelium of the former mice (Figure 9 A to F). No alveolar fibrosis was observed. In addition, PAS staining of lung sections from PBS instilled mice showed almost no goblet cells while numerous goblet cells were present in the airway epithelium of chronically instilled mice (Figure 9 G to I). Finally, in accordance with the observations in BALF, an intense inflammation in peri-vascular and peri-bronchial areas containing numerous eosinophils was observed while no inflammation could be detected in PBS-treated mice (Figure 9 J to L).

Discussion

We previously demonstrated that mold spores from *A.alternata* and *C.herbarum*, when injected i.p. into BALB/c mice induce the production of specific IgG1 antibodies and strongly increase IgE serum levels [10]. Here we confirm and extend these results showing that mice chronically exposed only through the airways with *C. herbarum* spores or *A. alternata* extract in the absence of adjuvant mount a specific IgG1 and IgE antibody response and present an increased serum IgE concentration. However this strong non specific systemic Th2 response does not influence specific T cell responses and their production of IFN-γ, IL-4 and IL-13 induced by an i.p. immunization with an unrelated antigen (KLH).

Shang et al. [20] have elegantly shown that the non-specific polyclonal IgE production observed during a Th2 response was due to an enhanced production of IgE by already activated and committed B cells in the spleen and was dependent on IL-4. We believe that a similar mechanism might be responsible for the increase of IgE serum levels after inoculation of the mold spores or extracts.

Since IgE secretion is completely dependent on the activation of Th2 cells [21], these results suggest that T cells secreting Th2 cytokines are also activated. This activation was confirmed by the up-regulation of IL-13 mRNA expression in the lungs and the presence of high levels of IL-4, IL-5 and IL-13 in the BALF of chronically treated mice.

The lung inflammation induced in mice chronically instilled with *C.herbarum* spores and *A.alternata* extract was characterized by the presence in the BAL of numerous macrophages, lymphocytes, eosinophils and neutrophils. Our previous study demonstrated the presence of neutrophils in BAL of naive mice inoculated with mold spores and a strong up-regulation of MIP-1α, MIP-2 and eotaxin mRNA into the lungs while we noted that the accumulation of eosinophils into the lungs required a previous systemic sensitization [10]. However in the chronic settings of this study, neutrophils and eosinophils accumulated in the lungs without any previous systemic sensitization as also observed in human asthma [22]. Accumulation of eosinophils in the airways is a hallmark of allergic asthma [3] and these cells have a critical role in allergic airway remodeling [23]. Indeed, eosinophils are a main source of the pro-fibrotic factor TGF-β [24] and eosinophil-derived cationic proteins can induce the production of remodeling factors by lung epithelial cells [25]. A correlation has also been noticed between severity of asthma and number of neutrophils in the airway [26]. In severe asthma the

neutrophils are in an activated stage and therefore likely contribute to tissue damage [27] and neutrophils in the presence of IL-8 can increase the trans-basement membrane migration of eosinophils [28]. Moreover in a mouse model of airway allergy induced by *Aspergillus fumigatus* depletion of neutrophils resulted in reduced AHR and remodeling whereas conditional transgenic overexpression of CXCL1 in airway walls, leading to increased accumulation of only neutrophils in the lungs, worsened the allergic responses [2~~8~~9]. Activation of innate immunity and recruitment of neutrophils in response to *Aspergillus fumigatus* has been shown to be mediated by Dectin-1, an innate immune receptor expressed on alveolar macrophages and neutrophils. Dectin-1 specifically recognizes fungal β-glucans and triggers the inflammatory response and the release of MIP-1α, MIP-2 and TNF- α [30–32] . Since spores and extracts from *A.alternata* and *C.herbarum* are rich in β-glucans, a similar mechanism involving the recognition of these pathogen-associated molecular patterns by Dectin-1 might explain the accumulation of neutrophils in the airways of chronically treated mice. Another explanation could be the presence of LPS in our spore and extract preparations but this seems not very likely since the concentration of LPS in our extract was very low (between 3 and 6 U/ml analysed with the QCL-1000 LAL, Biowittaker, corresponding to 0.15-0.3 U per instillation).

Mice chronically instilled with *A.alternata* extract developed an AHR 3 weeks after the beginning of the treatment that remained constant during the whole experiment and did not return to baseline levels even 10 weeks after ceasing the instillations. AHR appeared at week 7 in mice treated with *C.herbarum* spores, remained stable two weeks after termination of the experiments and then declined slowly. These differences concerning the evolution of AHR could be explained by intrinsic differences between the two

molds but it is more likely that the quantity of available allergens was higher in the extract than in the spore inoculum. Moreover proteins instilled into the lungs are directly available to antigen presenting cells whereas spores need to be engulfed and processed.

In this study we also observed that a chronic treatment with the *C.herbarum* spores and *A.alternata* extract led to an increase of lung hydroxyproline, soluble collagen and fibronectin levels indicating that a remodeling process was occuring. This remodeling was confirmed by histological analysis showing a thickening of the airway epithelium, an accumulation of goblet cells and a strong subepithelial fibrosis. Subepithelial fibrosis is one of the predominant characteristics of airway remodeling observed in human asthma and is of great concern since several studies have linked the intensity of fibrosis to the severity of the disease in patients [33, 34] Fibrogenesis is thought to result from a chronic inflammation mainly characterized by the recruitment of eosinophils and activated lymphocytes. Upon stimulation these cells, as well as resident epithelial cells, release inflammatory mediators and growth factors, leading to the activation and proliferation of fibroblasts and to the accumulation of the extracellular matrix proteins [35]. Two cytokines detected in our model, IL-13 and TGF-β are well known pro-fibrotic mediators [36]. It is therefore likely that the remodeling process observed in our chronic model is linked to the production of both IL-13 and TGF-β while other non-detected factors might also be contributing to this response.

One of the striking result of this study was the development of the allergic response after a chronic i.n. instillation of mold spores or extracts in the absence of any systemic sensitization. This immune activation is in contrast to the induction of tolerance generally observed after exposure of the airway mucosa to soluble antigens by i.n.

administration [37]. Indeed i.n. exposure of mice to soluble OVA results in peripheral $CD4^+$ T cell unresponsiveness that leads to prevention of AHR, lung inflammation and specific IgE production [38]. However the same i.n. OVA exposure leads to a lung allergic phenotype and an abrogation of tolerance if mice are pre-sensitized with the use of adjuvant. In contrast to OVA, natural allergens as for instance *Aspergillus fumigatus* antigens or ragweed pollen extract have been shown to elicit robust allergic lung inflammation when inoculated into the airways of naive mice and this response was dependent upon a proteolytic activity necessary to bypass the tolerogenic response to inhaled antigens [39]. Indeed, natural allergens implicated in asthma are often either proteases or strongly associated with protease activity such as Der P1 [40] and Fel D1 [41]. Der P1's ability to induce proinflammatory cytokine release from respiratory epithelial cells is known to be mediated by activation of protease-activated receptor (PAR)-2 on these cells [40] Exogenous PAR-2 activation in parallel with OVA challenge enhances allergen-mediated AHR and airway inflammation [42]. Moreover, PAR-2 activation also leads to the release of mediators such as eotaxin, GM-CSF and metalloproteinases [43, 44]. *A.alternata* and *C.herbarum* extracts possess a high protease activity and are potent inducers of epithelial cell desquamation and production of pro-inflammatory cytokines (IL-6 and IL-8) which is strictly dependent on the protease activities of these extracts [45]. Our data suggest that PAR-2 activation through protease activity from mold allergens might explain the inhibition of tolerance and the stimulation of lung inflammation by the allergens tested. Further experiments will aim at analysing the importance of the extract protease activity in this model.

In conclusion, we have descibed in this study a mouse model of lung allergy that does not require any sensitization, uses relevant particulate or proteic allergen from non

invasive molds, induces allergic inflammation, AHR and subepithelial fibrosis. This model could be an interesting tool to dissect the mechanisms leading to the activation of allergic immune responses in the lungs and remodeling and for the design of new therapeutics.

Footnotes

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Legend to the figures

Figure 1: Polyclonal IgE synthesis induced by chronic i.n. instillations of *Alternaria alternata* extract and *Cladosporium herbarum* spores. BALB/c mice were chronically instilled i.n. with PBS, with 5 µg of *Alternaria alternata* extract or 2.10^5 spores of *Cladosporium herbarum* for 10 weeks. Twenty four hours after the last instillation mice were bled and total IgE levels were determined. Significant differences (** *P<0.05,* *** *P<0.01*) between values from extract or spores treated mice versus PBS treated mice.

Figure 2: Specific IgE and IgG1 antibody synthesis induced by chronic i.n. instillations of *Alternaria alternata* extract and *Cladosporium herbarum* spores. BALB/c mice were chronically instilled i.n. with PBS, with 5 µg of *A.alternata* extract or 2.10^5 spores of *Cladosporium herbarum* for 10 weeks. Twenty four hours after the last instillation mice were bled and specific IgE and IgG1 were determined. Data represent the mean ± SEM of the A_{450} values of 5 to 7 mice per group.

Figure 3: Chronic i.n. instillations of *Alternaria alternata* extract do not influence the T cell response induced by immunization with a model antigen. BALB/c mice were chronically instilled twice a week i.n. with PBS or with 5 µg of *A.alternata* extract during the entire experiment. Three weeks after the beginning of instillations, mice were immunized i.p. twice with 50µg of KLH emulsified in CFA or in Alum. Mice were boosted two weeks later with 50µg of KLH emulsified in IFA or in Alum respectively. One week after the KLH boost, spleen cells were restimulated *in vitro* for three days with medium only or medium supplemented with KLH or PWM. The concentrations of IFN-γ, IL-4 and IL-13 in supernatants were measured by ELISA.

Figure 4: Inflammatory cell recruitment in the lungs of *A.alternata* extract and *Cladosporium herbarum* spore-treated mice. BALB/c mice were chronically instilled i.n. with PBS, with 5 µg of *A.alternata* extract or 2.10^5 spores of *Cladosporium herbarum* for 10 weeks. (A) BALF was collected 24 h after the last instillation and the total cell number was determined. (B) Differential cell count of the inflammatory subpopulation in BALF 24 h after the last instillation. Data represent the mean ± SD of 5 to 6 individual mice per group.

Figure 5: Mice chronically instilled with *Alternaria alternata* extract or *Cladosporium herbarum* spore develop a sustained AHR to inhaled methacholine. BALB/c mice were chronically instilled i.n. for 10 weeks with PBS, 5 µg of *Alternaria alternata* extract or 2.10^5 spores of *Cladosporium herbarum*. P_{enh} were measured by whole body plethysmography after 3, 5, 7, 9 and 11 weeks. Results are expressed as means ± SEM of 5 to 7 mice per group.

Figure 6: (A) Chronic instillations of *Alternaria alternata* extracts induce the production of IL-13 mRNA in the lungs. BALB/c mice were left untreated (NT), chronically instilled i.n. with PBS or with 5 µg of *Alternaria alternata* extract for 10 weeks. At indicated time points (24 hours after the last instillations) total RNA was extracted from the lungs and transcriptional levels of IL-13 were analysed by quantitative RT-PCR. Data represent the mean ± SD of triplicate assays. (B) Chronic instillations of *Alternaria alternata* extracts or *Cladosporium herbarum* spores induce an accumulation of IL-13 into the BALF. BALB/c mice were chronically instilled i.n. with PBS, with 5 µg of *Alternaria alternata* extract or 2.10^5 spores of Cladosporium *herbarum* during 10 weeks. 24 hours after the last instillation BALF were collected and IL-13 concentrations in BALF were analysed by ELISA. Significant differences (***

P<0.01) between values from extract or spores treated mice versus PBS treated mice. There was no significant difference between extract versus spores-treated mice.

Figure 7: Quantification of IL-4, IL-5, IL-6, IL-10 and TGF-β concentrations in the BALF of mice chronically instilled with *Alternaria alternata* extract. BALB/c mice were chronically instilled i.n. with PBS or with 5 µg of *Alternaria alternata* extract during 10 weeks. BALF were collected 24 h after the last instillation and IL-4, IL-5, IL-6, IL-10 and TGF-β concentrations were determined by ELISA. Significant differences (** *P<0.05*, *** *P<0.01*) between values from extract-treated mice versus PBS-treated mice for IL-4, IL-5, IL-6 and TGF-β. Non significant (*NS*) for IL-10.

Figure 8: Levels of hydroxyproline in lung homogenates, soluble collagen in supernatents of lung homogenates and fibronectin in BALF of BALB/c mice chronically instilled for 10 weeks with PBS or *Alternaria alternata* extracts. (** *P<0.05*).

Figure 9: Histopathological changes in the lungs of mice chronically instilled with *Alternaria alternata* and *Cladosporium herbarum.* BALB/c mice were instilled i.n. during 10 weeks with PBS (left column), *Alternaria alternata* extracts (middle column) or *Cladosporium herbarum* (right colum). The fist two rows show lung sections stained with Masson trichrome. The third row shows lung sections stained with PAS and the last row lung sections stained with HES.