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# Investigation of inflammatory and allergic responses to common mold species: Results from in vitro experiments, from a mouse model of asthma, and from a group of asthmatic patients

M. Vincent[1] (iD) | P. Percier[1] | S. De Prins[1] | K. Huygen[1] | G. Potemberg[2] | E. Muraille[2,3] | M. Romano[1] | O. Michel[4] | O. Denis[1]

[1]Scientific Service Immunology, WIV-ISP, Bruxelles, Belgium

[2]Unité de Recherche en Biologie des Microorganismes, Laboratoire d'Immunologie et de Microbiologie, NARILIS, Université de Namur, Namur, Belgium

[3]Laboratoire de Parasitologie, Faculté de Médecine, Université Libre de Bruxelles, Bruxelles, Belgium

[4]Clinic of Immunology and Allergology, CHU Brugmann, Université Libre de Bruxelles, Bruxelles, Belgium

**Correspondence**
Olivier Denis, Scientific Service Immunology, WIV-ISP, Bruxelles, Belgium.
Email: odenis@wiv-isp.be

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## Abstract

Most studies on molds focus on *Alternaria alternata* and *Aspergillus fumigatus*. Here, we report on inflammatory and allergenic properties of more typical indoor species *Aspergillus versicolor*, *P. chrysogenum*, *C. cladosporioïdes*, and *C. sphaerospermum* that were compared to *A. alternata* and *A. fumigatus*. In a mouse model, after intranasal instillation, *A. alternaria*, *A. versicolor*, and *C. sphaerospermum* induced the early recruitment of neutrophils and the strong expression of inflammatory markers in the bronchoalveolar lavages fluids. *A. fumigatus* also induced the early accumulation of neutrophils but with lower levels of inflammatory markers. Chronic treatment induced variable response according to species: *P. chrysogenum* and *A. fumigatus* appeared strong pro-allergenic inducers compared to *A. alternata* and *C. sphaerospermum* while *A. versicolor* and *C. cladosporioides* induced a mixed pro-allergenic/pro-inflammatory response. In mold-sensitized asthmatics, mold-specific Immunoglobulin E (IgE) were detected with an in-house dot-blot assay. *A. fumigatus* and *A. alternata* were the most frequent sensitizers. Altogether, *P. chrysogenum*, *P. brevicompactum*, *C. sphaerospermum*, and *C. cladosporïoides* were the "major sensitizer" (defined as the strongest response against a single mold species) for almost 30% of the asthmatics. These results show that, not only *A. alternata* and *A. fumigatus*, but also indoor species have strong inflammatory and allergic properties and a harmful potency.

**KEYWORDS**
asthma, dot-blot assay, indoor molds, inflammatory properties of molds, mold sensitization, mouse model of asthma

## 1 | INTRODUCTION

Molds are ubiquitous filamentous fungi able to colonize multiple environments. The majority of mold species are saprophytes, growing on most substrates, and recycling organic materials if enough moisture is available. So far, more than 112 different mold species have been recognized as important source of allergens that can cause respiratory diseases.[1]

Fungal spores are metabolically inactive and can tolerate environmental stress. Depending on the species, the size of mold spores ranges from 2 to 50 μm allowing their deposition into the airways. The external layer of the spores and the mycelium is a complex matrix structure

with innate immune system stimulating properties such as the β-1,3 glucans recognized by C-type lectin receptors (dectin-1 and 2, Mincle and mannose receptors) expressed on phagocytic cells, dendritic cells (DCs), neutrophils, and epithelial cells. The engagement of these C-type lectin receptors triggers the activation of intracellular signaling pathways leading to the activation of inflammasomes and promotes inflammatory responses critically required to control fungal infections. Indeed, the activation of dectin-1 at the surface of DCs by *A. fumigatus* induces the respiratory burst and the production of inflammatory mediators, including tumor necrosis factor α (TNF-α), various interleukins (IL-1β, IL-6, IL-23), chemokines ligands (CCL-2 and CCL-3), and chemokine (C-X-C motif) ligand 1 (CXCL-1).[2] In addition, dectin-1 knock-out mice have an increased mortality rate to an *A. fumigatus* challenge due to a poor neutrophil recruitment and an impaired fungal killing.[3] These findings show that C-type lectin receptors can activate innate immune cells, the first line of defense, and also modulate the development of the adaptive immunity through the differentiation of T-helper 1 (Th1) and T-helper 17 (Th17) cells, two critical cell subsets to mount a protective immune response against pathogenic fungi.[4-6] These responses have been widely investigated with the pathogenic *C. albicans* and *A. fumigatus* but the relative contribution of C-type lectin receptors in the development of immune responses against nonpathogenic molds as for instance *Penicillium spp*, *Cladosporium spp* or *A. alternata* is lacking and the mechanisms involved are not formally identified yet.

The fungal cell wall is also composed of glycoproteins with allergenic properties.[7] In addition, mold allergens are also found in enzymes released in the external environment during the germination process.[8,9] The lung epithelium and the dendritic cells present in the lung can recognize these allergens which initiates the allergenic process. Various signaling pathways, characterized by the secretion of IL-25, IL-33, and the activation of different innate immune cells, contribute to generate a pro-T-helper 2 environment in the lungs. This ultimately leads to the activation of nonprotective allergen-specific cells predominantly secreting characteristic Th2 cytokines such as IL-4, IL-5, and IL-13.[10,11] These cytokines induce IgE production, cause eosinophilia, stimulate mast cells, promote leukocytosis, and provoke airway hyper reactivity. This immune activation in asthma patients leads to repeated attacks of breathlessness, cough, and wheeze occurring secondary to broncho-constriction in the setting of airway hyper-responsiveness, mucus hyper-secretion, and airway remodeling.[12] However, in many patients with severe asthma, the lung inflammatory status is more heterogeneous and characterized by the presence of neutrophils and a production of IL-17. Therefore, it has been postulated that IL-17 may also play an active role in severe forms of the disease.[13-15]

*Alternaria alternata* and *Cladosporium herbarum* are the most common outdoor mold species. A large European study in asthmatics showed that the prevalence of sensitization to these two species increased significantly with increasing asthma severity.[16] In addition, exposure to airborne spores of *A. alternata* represents a risk factor for respiratory failure underlining the association between asthma severity and sensitization to this species.[17]

Mold spores can likewise be present in the indoor environment, due to some housing activities or inhabitant behaviors, but

the association between their presence and adverse health effects is controversial.[18,19] Choi et al.[20] found no association between the presence of ergosterol, β-glucans, or culturable molds and indoor dampness with asthma and allergic diseases symptoms of children. Other work found a positive association between respiratory adverse effects including asthma development or exacerbation and residential dampness but not with quantitative mold assessments.[21,22] Finally, other studies indicated that exposure to some mold species commonly contaminating damp dwellings were associated with increased exacerbations and asthma development in children.[23,24] In these settings, *A. versicolor*, *P. chrysogenum*, *C. cladosporioïdes*, and *C. sphaerospermum* are among the most common airborne and dustborne species isolated indoors.[25-28]

To document the hazardous nature of indoor mold species and define their allergenic potential, we analyzed the activating properties of *C. cladosporioides*, *P. chrysogenum*, *C. sphaerospermum*, and *A. versicolor* and compared them to those of the outdoor mold *A. alternata* and the classical allergenic mold *A. fumigatus*. The cytokine secretion profile was studied after bone marrow-derived dendritic cells (BMDCs) stimulation with the spores of the six species. We also analyzed both acute and chronic immune response in an experimental model of asthma established in mice chronically instilled intra-nasally with mold spores. In parallel, the prevalence of sensitization to 10 mold species frequently isolated in Western Europe[25,28] was estimated in a population of mold-sensitized asthmatics. Results underline the noxiousness of *A. fumigatus* and *A. alternata* but also of exposure to indoor mold species.

## 2 | MATERIALS AND METHODS

### 2.1 | Mold strains, spores and extract preparations

*Alternaria alternata* (strain 18586), *C. cladosporioides* (strain 9072 and 6032), *P. chrysogenum* (strain 1240), *C. sphaerospermum* (strain 22974), and *A. versicolor* (strain 18884) were obtained from the Belgian Co-ordinated Collections of Micro-organisms/Institut d'Hygiène et d'Epidémiologie-Mycologie (BCCM™/IHEM) from the Wetenschappelijk Instituut Volksgezondheid-Institut Scientifique de Santé Publique (WIV-ISP, Brussels, Belgium). *A. fumigatus* (strain AF293) was obtained from the American Type Cell Culture (ATCC—Manassas, VA, USA). These molds were cultured at 27°C (20°C for *A. versicolor* and *A. fumigatus*) on potato dextrose agar

(BD Difco—Franklin Lakes, NJ, USA) plates, for 2 weeks before gently harvesting the spores with a cell scraper. Spores were diluted in phosphate-buffered saline (PBS) and counted with a hemocytometer.

Aqueous extracts from *A. alternata*, *P. chrysogenum*, *A. fumigatus*, and *C. sphaerospermum* were purchased from Greer laboratories (μg/mL; Greer Laboratories — Lenoir, NC, USA). *A. versicolor* and *C. cladosporïoïdes* extracts were prepared from mycelial mats cultured during 3 weeks in liquid Czapek media. Mats were grinded in liquid nitrogen as described by Achatz et al.[29] Protein concentration was measured according to the Bradford method, and extracts were stored at −20°C.

## 2.2 | In vitro BMDCs stimulations

Bone marrow-derived dendritic cells (BMDCs) were prepared as described.[30] Briefly, C57BL/6 mice were sacrificed and the bone marrow was extracted from femurs by flushing with PBS. Cells were cultured (5% $CO_2$ at 37°C) in T75 flask at a density of $4 \times 10^6$ cells per plate in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), $5 \times 10^{-5}$ mol/L β-mercaptoethanol, gentamycin, non-essential amino acids, sodium pyruvate, and glutamax (Thermo Fisher Scientific—Waltham, MA, USA) and mouse recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) 10 ng/mL (Immunotools—Friesoythe, Germany). The culture medium was replenished after 3 days. BMDC's were collected after 7 days, seeded in 96-well plates ($2 \times 10^5$ cells/well) and cultured for 24 hours with either lipopolysaccharide (LPS; 100 ng/mL) or curdlan (100 μg/mL) or zymosan (100 μg/mL) or various mold spores ($2 \times 10^5$ spores, except for *A. alternata* $2 \times 10^4$ spores).

## 2.3 | Animals and immunizations

C57BL/6 mice were obtained from the Elevage Janvier (Le Genest Saint Isle, France) or from the breeding facilities of the WIV-ISP and maintained under standard laboratory conditions. All experiments were performed in accordance with the local ethics committee and were approved under the file number 120724-01. Female mice aged 10-12 weeks at the beginning of experiments (five mice per group unless otherwise stated) were instilled intranasally (i.n.) with $10^6$ mold spores on three consecutive days (on Monday, Tuesday, and Wednesday) to study early responses. To study chronic responses, the same protocol was used during the first week followed by two instillations per week (on Mondays and Fridays) during 4 weeks and a final instillation the fifth week, on Monday. As instillation of $10^6$ *A. alternata* spores induced severe cachexia (related to strong TNF-α production), a lower concentration of $10^5$ spores was used.[31] The control mice were instilled with PBS only. Mice were anesthetized with isofluran (Isoflo, Abbott Laboratories—North Chicago, IL, USA) before instillations. Mice were killed and sampling was performed 24 hours after the last instillation.

To induce a polyclonal IgE production, mice were immunized twice by intra-peritoneal (ip) injections of $2 \times 10^6$ spores diluted in PBS at 1-month interval and sera were collected 15 days after the second injection.[32]

## 2.4 | Bronchoalveolar lavages

Bronchoalveolar lavage (BAL) fluids were collected as described before.[32] Total cell counts were determined with a hemocytometer, differential cell counts were obtained by examining at least 300 cells on cytospin slides stained with Diff-Quick (Dade Behring—Deerfield, IL, USA) and expressed as cells/mL.

## 2.5 | Cytokine detection in supernatants and BAL fluids

The concentration of TNF-α, IL-1α, and IL-1β, IL-6, IL-12, and IL-23 produced in vitro by BMDC's was determined by ELISA (Ready-SET-Go ELISA—eBiosciences, San Diego, CA, USA). The concentration of IL-4, IL-5, IL-13, and IL-33 was measured in BAL fluids by ELISA (R&D systems, Minneapolis, MN, USA). The concentration of TNF-α, IL-6, and IL-17 was determined by Cytokine Beads Array (BD Pharmingen—San Diego, CA, USA). Samples were acquired on a FACScalibur and analyzed with the Flow Cytometric Analysis Program array software (BD Biosciences—Franklin Lakes, NJ, USA).

## 2.6 | Total mouse IgE determinations

Total serum IgE concentrations were determined using a sandwich ELISA as described.[32]

## 2.7 | mRNA chemokine and cytokine quantification

Total RNA was extracted from homogenized lung cells with Tri-reagent (Sigma-Aldrich—Saint Louis, MO, USA), 24 hours after the last instillation. cDNA was synthesized using the GoScript Reverse Transcription System (Promega—Madison, WI, USA). RT-qPCR was performed on the Stratagene Mx3000P (Agilent Technologies—Santa Clara, CA, USA) using a GoTaq qPCR Master Mix with BRYT Green (Promega). Each RT-qPCR amplification was performed in duplicate under the following conditions: 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. The list of forward and reverse primers used in this study is shown in Table S1. Hydroxymethylbilane synthase (HmBS) mRNA was used as reference housekeeping gene for normalization. The level of target mRNA, relative to the mean of the reference housekeeping gene, was calculated as previously described.[33]

## 2.8 | Evaluation of histological changes in the lungs

For histology analysis, lungs were collected 24 hours after the last instillation and fixed by tracheal infusion of formalin. Tissues were kept in formalin for 2 days and then embedded in paraffin according to a protocol previously described.[31] For microscope examination, tissues were sliced and then colored with hematoxylin-eosin-safran for the detection of lung inflammation and with periodic acid Schiff for the detection of mucus produced by goblet cells. The lung preparation steps for histology analysis were conducted in Unité de Recherche

en Physiologie Moléculaire (URPhyM), NARILIS, Université de Namur (laboratory of Pr. Y. Poumay).

## 2.9 | Asthmatic subjects

Asthmatic patients were enrolled in the Immuno-allergology Unit of Centre Hospitalier Universitaire (CHU) Brugmann (Brussels). The group consisted of 32 asthmatics, with a positive skin prick test (SPT) and/or CAP (cellulose acetate membrane precipitin) test for *A. fumigatus* and/or *A. alternata* and/or *P. chrysogenum* and/or *C. herbarum*. Asthma was diagnosed according to the Global Initiative for Asthma (GINA) guidelines.[34] SPTs were carried out using a standardized procedure with reagents from Stallergènes (Antony—France). Positive (histamine and codeine) and negative (allergen diluent) controls were used. Weal size was measured after 20 minutes, and the result was considered positive if the weal was at least 4 mm greater than the negative control. CAP tests were performed in the Immunology laboratory at CHU Brugmann with the Phadia 1000 FEIA analyzer (Thermo Fisher Scientific) against *A. fumigatus*, *A. alternata*, *P. chrysogenum*, and *C. herbarum*. According to the manufacturer, the test has a limit of detection of 0.1 kU/L and a positivity threshold of 0.35 kU/L. A written informed consent was obtained for each patient, and the study protocol was approved by the local Ethics Committee (CE2011/71).

## 2.10 | Human mold specific IgE detection

A dot-blot assay was developed in our laboratory for the detection of specific IgE.[35] Briefly, mold extracts, diluted in borate buffer (pH 10), were coated on a nitrocellulose membrane (Amersham Protran 0.45 NC; GE Lifesciences—Chicago, IL, USA; 5 and 50 µg/mL for commercial and in-house extracts, respectively) using a 96-well dot-blot system (Minifold®I Dot-Blot System; Whatman Saint Louis, MO, USA). The membrane was removed from the device and blocked in 1% bovine serum albumin (BSA; Sigma-Aldrich) in tris-buffered saline (TBS) for 1 hour at room temperature before being incubated with patient's sera (dilution 1/5 in TBS-Tween20 0.1%-BSA 0.1%. 1.5 hours. Room temperature). The membrane was then incubated for 1.5 hours at room temperature with rat monoclonal anti-human IgE coupled to horseradish peroxidase (HRP; LO-HE 17-HRP & LO-HE 10-HRP—Institute of Experimental and Clinical Research, Université Catholique de Louvain, Belgium) diluted 1/5000 in TBS-Tween20 0.1%-BSA 0.1%. Between each incubation step, excess antibodies were removed by three successive washes with TBS for 3 minutes and then three times with TBS-Tween20 0.05% for 5 minutes and then again three times with TBS for 3 minutes. The membrane was developed by addition of Amersham ECL Prime reagent (GE Lifesciences), and readings were performed on a bioanalyzer CCD-Camera (LAS 4000Mini; GE Lifesciences) and analyzed with the Image Quant TL software (GE Lifesciences). Two positive controls were used. The Non-WHO Human anti-*Aspergillus fumigatus* IgE Reference Serum (Product number 78/545 National Institute for Biological Standards and Controls—NIBSC, Hertfordshire—UK; concentration 25.6 kU/L measured by ImmunoCAP) and the serum of a volunteer with documented sensitizations to several mold species. Two sera that had shown no reactivity against any mold of the panel were used as negative controls. The background was estimated based on the signal from an uncoated membrane (sample dilution buffer). The results, obtained as pixel counts, were then converted into indexes to standardize their expression with the following formula:

$$\text{INDEX} = \frac{(\text{pixel count for the tested serum} - \text{background})}{(\text{pixel count for the first negative control} - \text{background})}.$$

For each mold extract, positivity thresholds were established from 43 serial analyses of the second negative control serum. Positivity thresholds were expressed as the mean index response obtained for each extract +3 standard deviations. For each positive serum, the "major sensitization," defined as the response against a single extract showing the highest difference as compared to the variation of response of the negative control was calculated with the following formula:

$$\Delta \text{ for each extract} = \frac{(\text{experimental serum index} - \text{index of the second negative control serum})}{\text{standard deviation of the second negative control serum}}.$$

This formula was used because it not only considers the gap between the response against the serum and the negative control for a certain mold extract but also the variation of the response. The positivity threshold of the dot-blot was calculated based on a dilution curve of the Non-WHO Human Reference Serum anti-*Aspergillus fumigatus* on a *A. fumigatus* extract coated membrane and is of 0.418 kU/L. As no reference serum exists for other mold species, we had to assume that the sensitivity was comparable for other species.

## 2.11 | Statistical analysis

Mann-Whitney test was used for comparisons between two groups (mold spores vs RPMI/PBS). Cell recruitment in the BAL fluids was compared between mold species with one-way ANOVA and Kruskal-Wallis test. Correlation between neutrophils count and CXCL2 expression was calculated with the Spearman correlation test. Significance was assumed at a $P$ value below .05. Data were obtained from at least two separate experiments. Analysis was performed with GraphPad Prism version 6.01.

## 3 | RESULTS

### 3.1 | Inflammatory responses induced by the mold spores

The in vitro inflammatory properties of spores from indoor mold species (*C. cladosporioides*, *P. chrysogenum*, *C. sphaerospermum*, and *A. versicolor*) were compared with two frequently studied species, *A. alternata* and *A. fumigatus*. Murine BMDCs were cultured in the presence of spores from these six species, and the production of inflammatory cytokines and chemokines released in the culture supernatant was analyzed. As shown in Figure 1, all tested mold species except *A. versicolor* induced a substantial production of TNF-α.
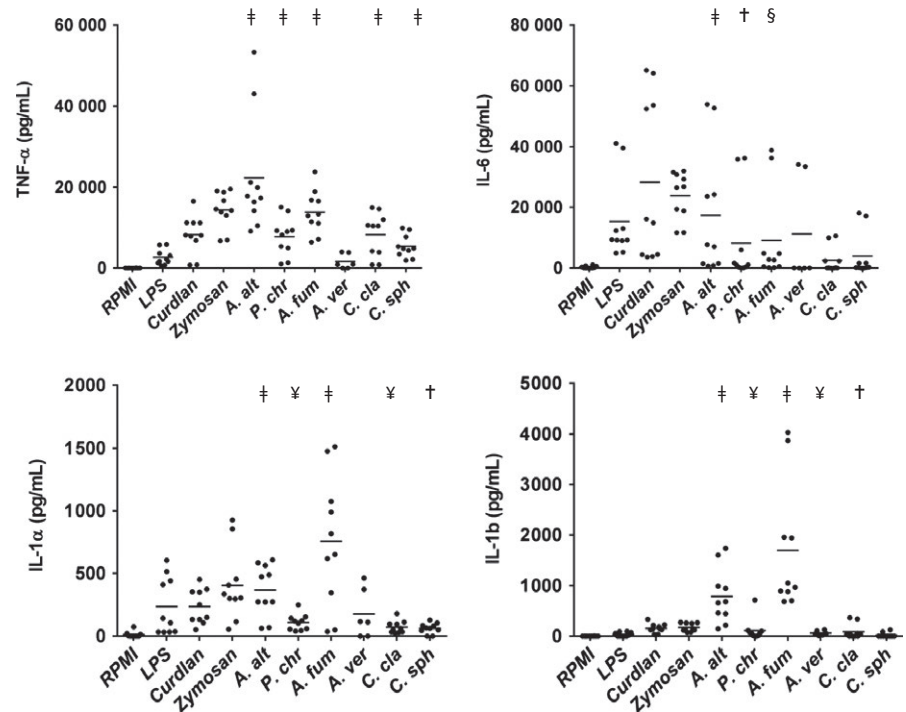
**FIGURE 1** In vitro inflammatory cytokine production by BMDCs in response to mold spores. $2\times10^5$ BMDCs from C57BL/6 mice were incubated with RPMI medium alone, LPS (100 ng/mL), zymosan (100 µg/mL) or various mold spores ($2\times10^5$ spores except $2\times10^4$ spores for *A. alternata*). The concentration of each cytokines was measured in culture supernatants after a 24-h culture at 37°C. Results represent cytokine concentrations from five mice tested in duplicate (†$P<.05$, §$P<.01$, ¥$P<.001$, ‡$P<.0001$)

*A. fumigatus* and *A. alternata* induced also a very significant and strong production of IL-6, IL-1α, and IL-1β while other species elicited lower and variable production of these cytokines (Figure 1). None of the six tested species stimulated the production of IL-12. *A. fumigatus* and *A. alternata* triggered a significant albeit low production of IL-23 (data not shown).

The acute inflammatory properties of these species were also examined in vivo in C57BL/6 mice, 24 hours after three (once a day) intranasal instillations of the spores. In the BAL fluids, all mold spores induced a recruitment of macrophages. *A. versicolor*, *C. sphaerospermum*, *A. alternata,* and *A. fumigatus* elicited stronger neutrophil recruitment than *C. cladosporïo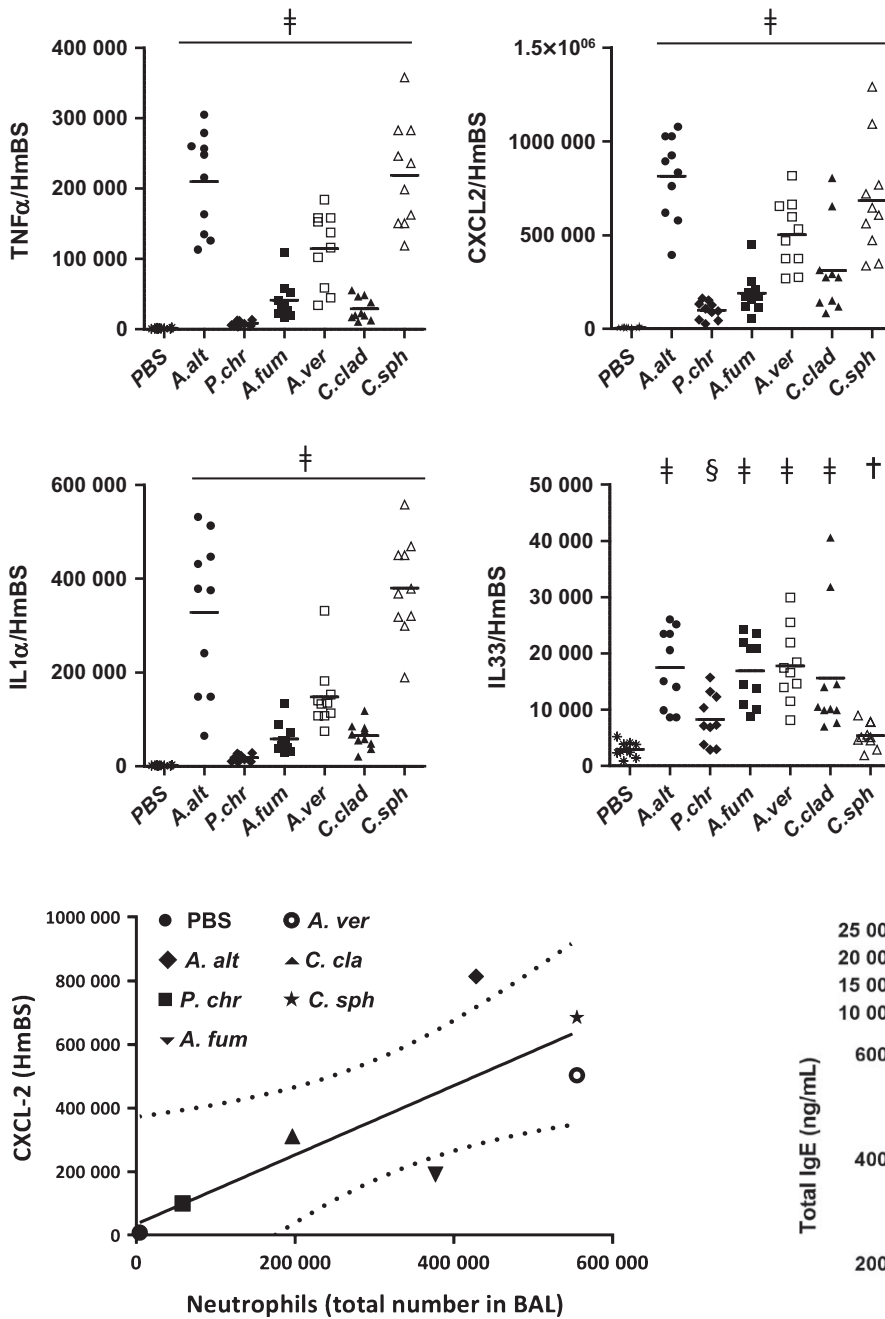ïdes* while *P. chrysogenum* only induced a minimal accumulation (Figure 2). The patterns of TNF-α, CXCL-2, and IL-1α mRNA lung expression were similar. These three cytokines were strongly induced by *A. alternata*, *C. sphaerospermum*, and *A. versicolor*. Other species such as *A. fumigatus*, *C. cladosporioïdes*, and *P. chrysogenum* elicited low but significant responses of these cytokines (Figure 3). The recruitment of neutrophils in BAL fluids was positively correlated with the lung expression of CXCL-2 ($r^2=.6597$; $P=.02$; Figure 4) indicating a match between cytokines and cells responses.

The expression of IL-33 in the lungs was also investigated. We found that the spores of the six mold species upregulated lung IL-33 with *P. chrysogenum* and *C. sphaerospermum* spores inducing a lower expression than spores of the four other species (Figure 3).
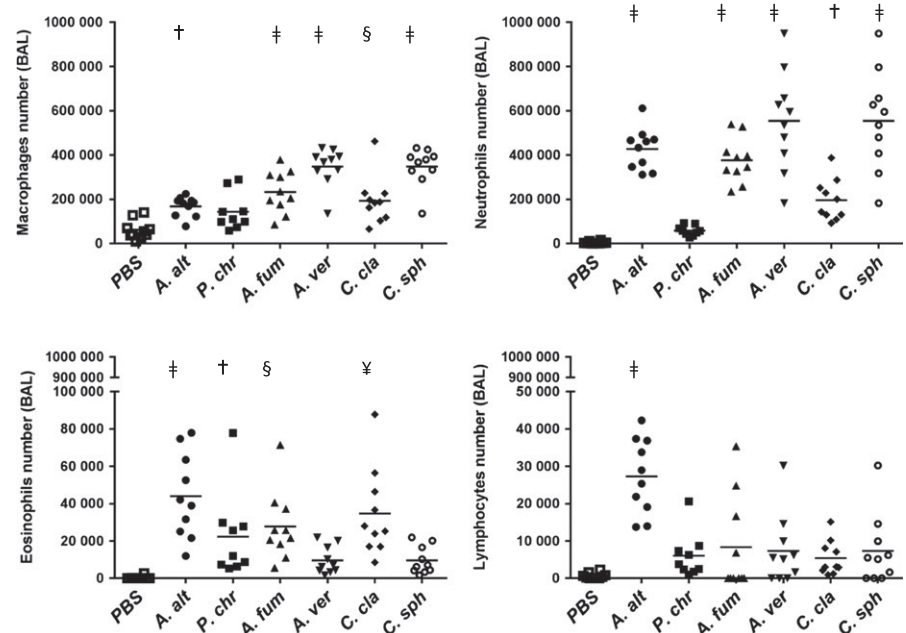


**FIGURE 2** Early inflammatory cell recruitment in the bronchoalveolar lavage fluids of mold spore-treated mice. C57BL/6 mice were instilled daily during 3 days with PBS or various mold spores ($10^6$ spores except $10^5$ spores for *A. alternata*), and the BAL fluids were collected 24 h after the last instillation. Results show the cell counts, per cell type for each mold species and were obtained from two independent experiments with five mice per group (†$P<.05$, §$P<.01$, ¥$P<.001$, ‡$P<.0001$)

**FIGURE 3** Analysis of the early cytokine/chemokine lung mRNA expression after stimulation with mold spores. C57BL/6 mice were instilled daily during 3 days with PBS or various mold spores ($10^6$ spores except $10^5$ spores for *A. alternata*). Twenty-four hours after the last instillations, total RNA was extracted from the lungs, and transcriptional levels of the indicated cytokines were analyzed by quantitative RT-PCR. Results are pooled from two independent experiments with five mice per group. Significant differences between mold-treated mice vs control PBS-treated mice (†$P<.05$, §$P<.01$, ¥$P<.001$, ‡$P<.0001$)
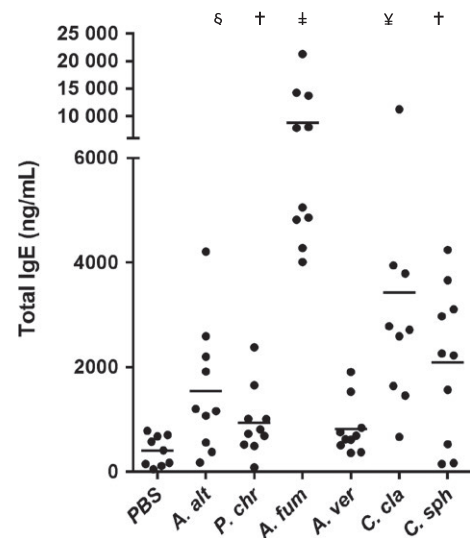


**FIGURE 4** Analysis of the correlation between neutrophil recruitment and CXCL2 expression in BAL fluid. The Spearman correlation test was used to study the correlation between neutrophil recruitment in the BAL fluids and the level of lung CXCL2 expression after three daily instillations of the indicated mold spores. Values plotted on the graph represent average counts from two independent experiments with five mice per group. Neutrophil recruitment and CXCL2 expression after mold stimulation were positively correlated ($r^2=.6597$; $P=.02$)



**FIGURE 5** Detection of total serum IgE levels induced by mold spore immunization. C57BL/6 mice were immunized intraperitoneally twice at 1-month interval with $2×10^6$ mold spores. Mice were bled 15 days after the boost. Data are from two independent experiments with five mice per group. Significant differences between IgE values of mold-treated mice vs control PBS-treated mice (†$P<.05$, §$P<.01$, ¥$P<.001$, ‡$P<.0001$)

## 3.2 | Allergic Th2 responses induced by the mold spores

As shown in Figure 5, all species except *A. versicolor* induced significant IgE serum levels. *A. fumigatus* and *C. cladosporïoides* generated the strongest responses. Cellular recruitments were analyzed in the BAL fluid 24 hours after the last instillation of a 5-week exposure scheme with the spores. Compared to the acute inflammatory response analyzed 24 hours after three intranasal instillations, a chronic

treatment during 5 weeks led to an additional recruitment of eosinophils. *A. fumigatus*, *P. chrysogenum*, *A. versicolor*, and *C. cladosporïoides* induced a stronger recruitment of eosinophils than the two other species. *A. alternata* and *C. sphaerospermum* recruited more neutrophils than the other species and led to a balance between neutrophils and eosinophils. In addition to neutrophils and eosinophils, macrophages and lymphocytes were also recruited in the BAL fluids of mice treated with these six species (Figure 6).

In parallel, the expression of five Th2-related cytokines and chemokines, IL-4, IL-5, IL-13, CCL-11, and CCL-24 was strongly increased in the lungs of mice treated with all six mold species. *A. alternata* induced less CCL-11 than the other species while *C. sphaerospermum* had a tendency to produce less IL-4. The expression of IFN-γ was also quantified (Figure 7) and the ratio IL-4/IFN-γ calculated (Supporting Information). Results showed that *P. chrysogenum* was the species with the highest IL-4/IFN-γ ratio while *A. alternata* had the lowest. The IL-4/IFN-γ ratio of the four other species was intermediate.

Lung mRNA expression and BAL fluid concentrations of IL-17 and TNF-α are shown in Fig. S2. *C. cladosporïoides* and *C. sphaerospermum* were the main stimulators of TNF-α while *A. versicolor*, *C. cladosporïoides*, and *C. sphaerospermum* were the main inducers of IL-17. *A. alternata* was also shown to increase the concentration of TNF-α in the BAL fluid. Noteworthy, *A. fumigatus* induced neither TNF-α nor IL-17 during this chronic phase. *P. chrysogenum* also failed to induce a production of TNF-α.

A summary of all the findings obtained in the mouse model is presented in the Table S2.

To perform further analysis of the lung inflammation, an histopathological examination was conducted. Control PBS-treated mice showed no sign of inflammation. In contrast, mice instilled with the spores showed an important accumulation of inflammatory cells. *P. chrysogenum*, *A. fumigatus*, *A. versicolor*, and *C. cladosporïoïdes* induced a recruitment mainly in the peri-vascular and peri-bronchial areas with numerous eosinophils. An accumulation of large macrophages in the lung parenchyma was also observed. After exposure to *A. alternata* and *C. sphaerospermum*, inflammation was more diffuse and fewer eosinophils were observed. Noticeably, in mice exposed to *P. chrysogenum*, the structure of bronchial epithelium was not very well-preserved and muscular hypertrophy was detected. Periodic acid Schiff (PAS) staining shows an intense production of mucus in mice exposed to mold spores. Of note, this production appeared less important after exposure to *C. sphaerospermum* (Figure 8).

## 3.3 | Analysis of the sensitization pattern to mold species in a group of asthmatics

The presence of specific IgE against the 10 tested mold species was investigated in a group of asthmatic patients with positive SPT and/or CAP test to commercial mold extracts. We used a dot-blot assay developed in our laboratory and worked with in-house mold extracts or commercial preparations that both differ from those usually used for SPT and CAP tests. Among these 32 patients, we found 25 patients with IgE reactivities against at least one mold of the panel. IgE against *A. fumigatus* and *A. alternata* were the most common, detectable in more than 40% of the asthmatics. In contrast, IgE against *C. cladosporïoides*, *A. versicolor*, and *A. flavus* were less common (less than 10% of the asthmatics). IgE antibodies against the other mold species were present in 20%-30% of the sera (Figure 9).

Cross-reactions are frequent in immune responses against mold antigens and have been reported in the literature.[36,37] Our concern was therefore to attempt to identify, in the sera showing more than one positive response, the "major sensitization." It was defined as the antibody response with the highest difference as compared to the variation of response of the negative control. Using this definition, we



**FIGURE 6** Late inflammatory cell recruitment in the bronchoalveolar lavage fluids of mold spore-treated mice. C57BL/6 mice were instilled daily during 3 days and then twice a week during 4 more weeks with PBS or various mold spores ($10^6$ spores except $10^5$ spores for *A. alternata*). Mice were instilled again the next Monday and BAL fluids were collected 24 h. Results show cell counts per cell type for each mold species. Data are from two independent experiments with five or six individual mice (*A. versicolor*, *C. sphaerospermum*, and *C. cladosporioides* group) per group († $P<.05$, § $P<.01$, ¥ $P<.001$, ‡ $P<.0001$)
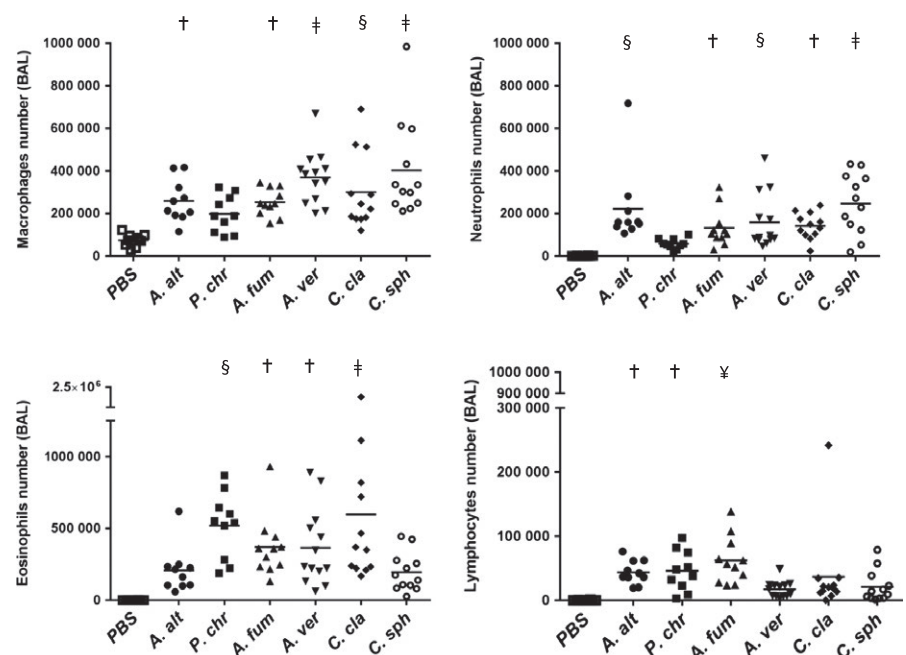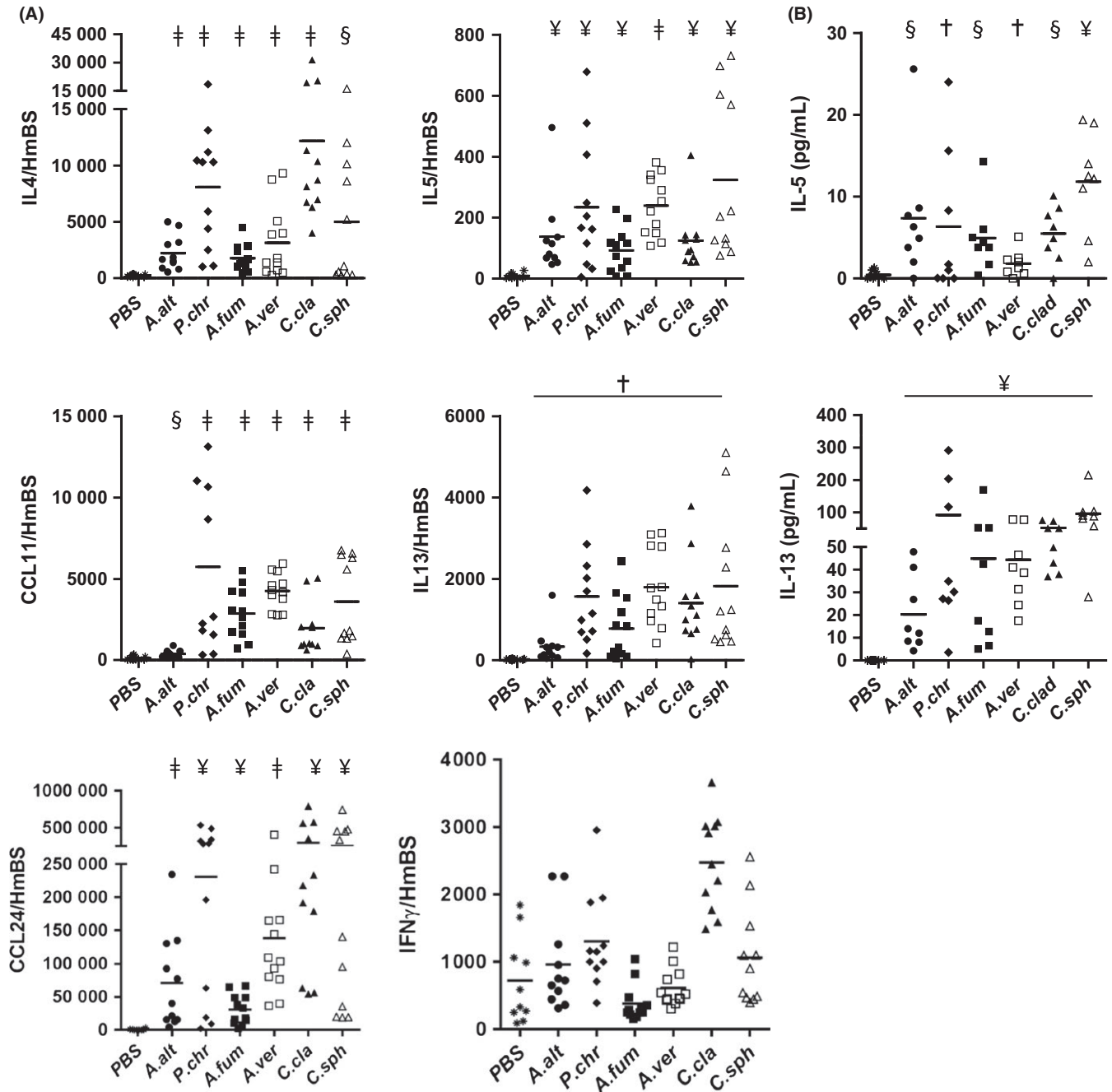
**FIGURE 7** Analysis of Th2-type cytokine production after a chronic stimulation with mold spores. C57BL/6 mice were instilled daily during 3 days and then twice a week during 4 more weeks with PBS or various mold spores ($10^6$ spores except $10^5$ spores for *A. alternata*). Mice were instilled again the next Monday. (A) The graphs show the lung transcriptional levels of the indicated Th2 cytokines and of IFN-γ analyzed by qRT-PCR 24 h after the last instillation. Results are from two independent experiments with five to six individual mice per group. (B) IL-5 and IL-13 concentrations in the BAL fluids 24 h after the last instillation. Results are from two independent experiments with five or six individual mice (PBS, *A. fumigatus*, *A. versicolor*, and *C. cladosporioides* group). Significant differences between mold-treated mice vs control PBS-treated mice (†*P*<.05, §*P*<.01, ¥*P*<.001, ‡*P*<.0001)

found that *A. fumigatus* was the most common "major sensitizer" (32% of the positive sera) followed by *A. alternata* in 28% of the asthmatics. *A. versicolor* was not identified as the "major sensitizer" in any of the asthmatic patients. Other mold species were less frequently identified as "major sensitizer." Although when taken together, *P. chrysogenum*, *P. brevicompactum*, *C. sphaerospermum*, and *C. cladosporioides* were the "major sensitizers" for almost 30% of the asthmatics.

## 4 | DISCUSSION

The association between building dampness and respiratory pathologies is established.[21,38,39] Additionally, recent studies are now pointing to a link between the presence of indoor molds and the development of asthma or the exacerbation of its symptoms.[23,24,40] Here, we have analyzed and compared the inflammatory and allergenic properties
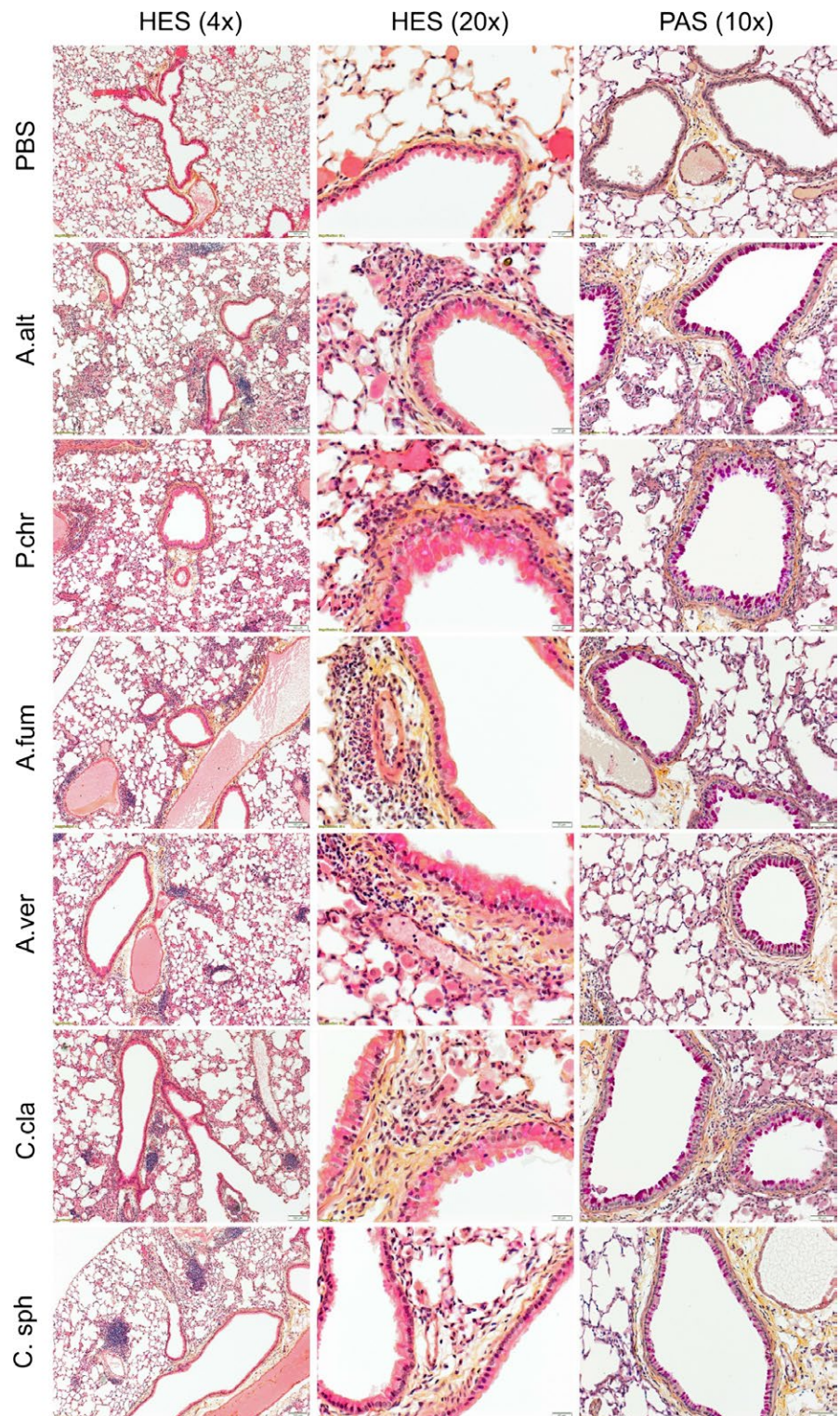
**FIGURE 8** Histopathological changes in lungs of mice chronically exposed to mold spores. C57BL/6 mice were instilled daily during 3 days and then twice a week during four more weeks with PBS or various mold spores ($10^6$ spores except $10^5$ spores for *A. alternata*). Mice were instilled again the next Monday and 24 h later, lungs were collected, fixed in formalin, and prepared for histological examination. Left and central panels show representative lungs sections after hematoxylin-eosin-safran staining. Right panel shows representative lung sections after periodic acid Schiff staining. Mold species are indicated on the left side of the figure

of six common mold species from indoor and outdoor environments. *A. alternata* is a typical outdoor mold while *A. fumigatus* is very common outdoors and indoors. *P. chrysogenum*, *A. versicolor*, *C. cladosporïoides*, and *C. sphaerospermum* are frequently found indoors, especially in damp dwellings.[18,25,27,28,41]

In vitro, spores from these six species elicited the secretion of inflammatory cytokines (TNF-α, IL-6, IL-1α and IL-1β) when cultured in the presence of murine BMDCs. The importance of C-type lectins in the recognition of β-glucans exposed in germinating spores has been clearly established.[42] In line with this, we found that this cytokine production was completely inhibited in MALT-1 (a caspase-like protease) deficient BMDCs. Indeed, cells that do not express MALT-1 are unable to form the CARD9-Bcl10-MALT1 complex downstream of the Dectin-Mincle receptors which subsequently hampers the activation of the NF-κB pathway (data not shown).
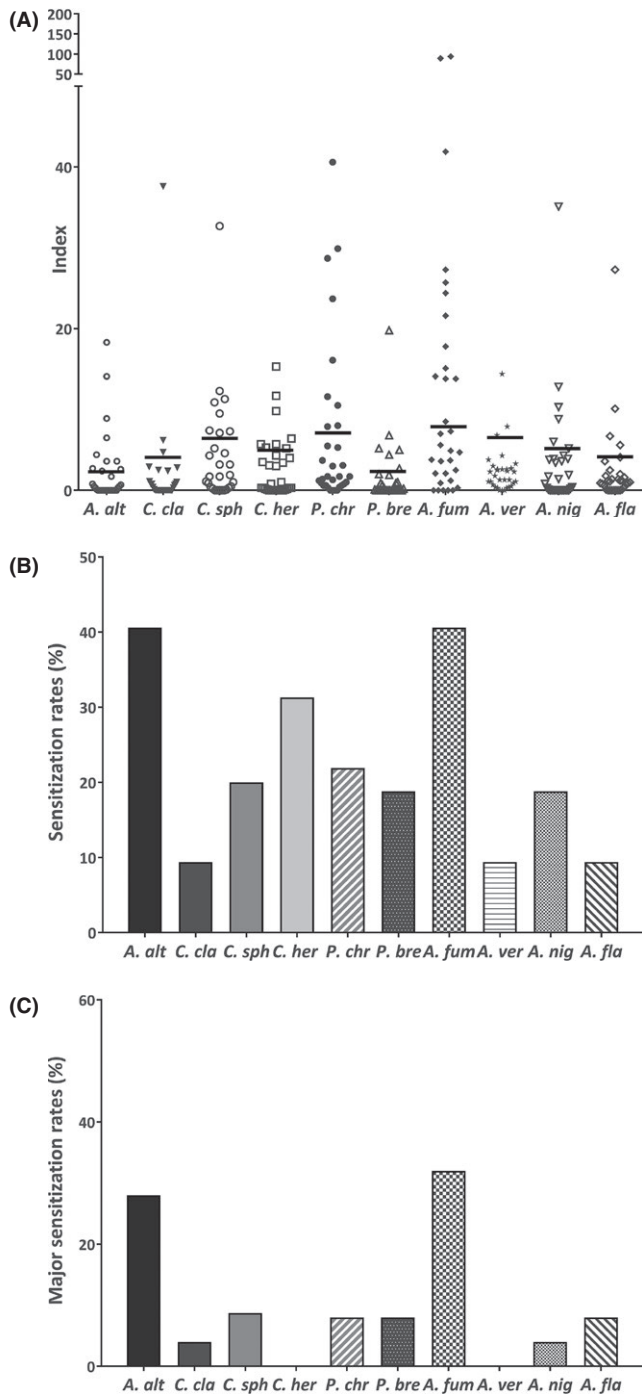
**FIGURE 9** Sensitization frequencies against a panel of indoor and outdoor molds in mold positive SPT/CAP asthmatic patients. (A) The presence of specific IgE against 10 different mold species was determined in a total of 32 sera from asthmatic patients with a positive SPT/CAP for molds. Horizontal bars represent, for each mold, the positivity threshold. This threshold, calculated for each mold extract, corresponds to the mean response +3 standard deviations of a negative serum. (B) Sensitization frequencies (%) for each mold of the panel (n=32). (C) Major sensitization frequencies (%) for each mold of the panel. For each serum, this major sensitization was defined as the response against a single extract showing the highest difference as compared to the variation of response of the negative control serum (n=25)

In vitro, TNF-α production was induced by the different spores to comparable levels except for *A. versicolor* that only induced a low TNF-α secretion. However, when instilled into the lungs, three species, *A. alternaria*, *A. versicolor*, and *C. sphaerospermum*, induced a stronger early upregulation of lung TNF-α expression than the three other species. The TNF-α produced in vivo by inflammatory dendritic cells has previously been shown to shape the immune response during fungal infection and to promote neutrophilic airway inflammation.[43] In our study, these three mold species also strongly upregulated the expression of lung CXCL-2 and induced an abundant early recruitment of neutrophils in the BAL fluids. Remarkably, for the six tested species, the recruitment of neutrophils in the BAL fluids was strongly correlated with the lung expression of CXCL-2. *A. fumigatus* also induced an early accumulation of numerous neutrophils into the lungs but with lower levels of lung TNF-α and CXCL-2. However, in vitro, *A. fumigatus* (and *A. alternata*) elicited a stronger secretion of IL-1α and IL-1β than the other species. In several models, IL-1 has been shown to be able to stimulate the production of various neutrophil chemo-attractants.[44,45] This axis might lie at the basis of the early neutrophil recruitment observed with *A. fumigatus* while *A. alternata* could trigger both TNF-α and IL-1 pathways. In contrast, *P. chrysogenum* spores only weakly recruited neutrophils in the BAL fluids and feebly upregulated the lung expression of inflammatory mediators indicating the weak inflammatory potential of this species.

In a previous report, we showed that spores from *A. alternaria* and *C. herbarum* injected in mice increased total IgE levels.[31,32] Here, we found that five of the six mold species tested induced a rise of the IgE serum concentrations. Only *A. versicolor* failed to induce any IgE increase. This lack of IgE increase was also observed by Mintz-Cole et al.[46] in their chronic model. An increase in serum IgE is usually indicative of a strong Th2 activation, secretion of IL-4 and/or IL-13 and STAT6 activation.[47]

We observed that spores of the six tested mold species led to an early upregulation of IL-33 expression when instilled in the lungs. This cytokine belongs to the IL-1 family and is rapidly upregulated in the lungs by some allergens e.g. *A. alternaria*.[48,49] Its biological activity is increased in an inflammatory environment, due to the release of proteases cleaving the full length IL-33 into its active form.[50] IL-33 initiates type-2 immune response through its binding to the receptor T1/ST2 present on innate lymphoid cells, basophils, mast cells, eosinophils, and subsets of Th2 cells.[50] Activated innate lymphoid cells vigorously produce IL-5 and IL-13 thereby creating an environment supporting the development of a Th2 response.[48]

Accordingly, a chronic treatment with spores from these six species led to the production of Th2 cytokines (IL4, IL-5, and IL-13) and a lung inflammation characterized by an accumulation of eosinophils in the BAL fluids. *P. chrysogenum* induced a strong recruitment of eosinophils with low neutrophil and macrophage numbers. For *A. fumigatus*, *A. versicolor*, and *C. cladosporïoides*, eosinophils outweighed neutrophils in the BAL fluids while *A. alternata* and *C. sphaerospermum* spores recruited more neutrophils than eosinophils. Histological analysis of the lungs confirmed that *P. chrysogenum*, *A. fumigatus*, *A. versicolor*, and *C. cladosporïoides* induced a recruitment of eosinophils in the

**(A)**

Inflammatory cytokines
Neutrophils
IL-17

C. cla
C. sph    A. alt         A. fum    P. chr
A. ver

Th2 cytokines
Eosinophils
IgE

**(B)**

Weak sensitizers in
asthmatics

A. alt
C. cla    P. chr    A. fum
A. ver    C. sph

Strong sensitizers in
asthmatics

**FIGURE 10** Classification of mold species according to their immunomodulatory properties. (A) Classification established based on results obtained from mouse model experiments; (B) Classification established based on results obtained from investigations on mold sensitization rates in a group of mold-sensitized asthmatics
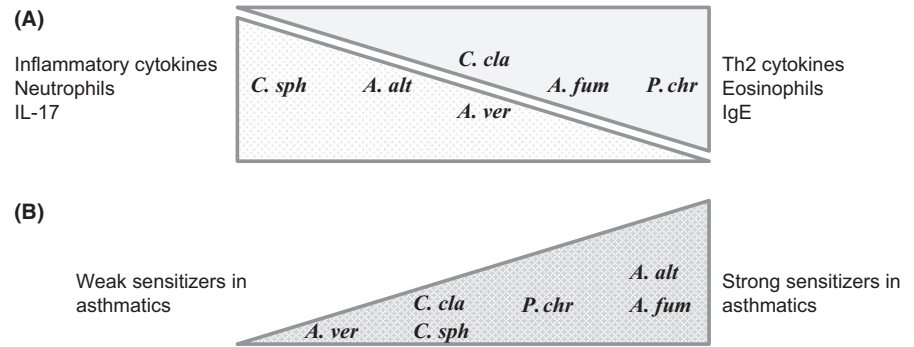
peri-bronchial areas and increased the mucus production (to a lesser extent for *C. shaerospermum*).

The ratio of IL-4/IFN-γ production in the lung also confirmed the strong pro-Th2 potential of *P. chrysogenum*. However, this ratio is only partially representative of the Th2 response and other cytokines (IL-5 and IL-13) and chemokines (CCL-11 and CCL-24) should also be considered together with IL-17.

In the studies of Mintz-Cole et al., *C. cladosporïoides* was described as a poor stimulator of the Th17 response due to a lack of beta-glucans present on the spore surface, whereas *A. versicolor* spores were shown to trigger dectin-1-dependent Th17 response leading to the development of a lung neutrophilic inflammation.[49,51] We also detected the production of IL-17 in the lungs and BAL fluids of *A. versicolor* stimulated mice but in our model this response was associated with a production of Th2 cytokines and a lung eosinophilia. A difference between mold strains could not explain this inconsistency as two other *A. versicolor* strains were tested with similar results (data not shown). However, mold cultures are not easily standardized and differences in the media or in the preparation of the spores or differences in the protocols might account for these discrepancies. Moreover, we found that *C. cladosporïoides* but also *C. sphaerospermum* were fully capable of stimulating lung production of IL-17, and thus, these three species induced mixed Th2/Th17 response when instilled chronically in the lungs of C57BL/6 mice. Th17 cells and their production of IL-23 are an integral part of the protective immunity against fungi, increasing antifungal responses by alveolar macrophages and neutrophils.[52] IL-17 can also upregulate the expression of CXCL chemokines leading to the recruitment of neutrophils, increase airway hyper-responsiveness and lies at the basis of the corticoid resistance of bronchial epithelial cells.[53-55] An imbalance between Th2 and Th17 activation could therefore turn protective responses to pathology/allergy.

In addition to the responses triggered by the six tested mold species in mice, we also evaluated the production of specific IgE in a population of asthmatic patients sensitized to molds. We used a panel of 10 molds species that are known to be frequently isolated in Western Europe.[25,28] In this population, we found IgE antibodies directed against all these mold species. In the context of mold sensitization, the cross-reactivity between various fungal allergens has been quite extensively reported. It generally involves fungi that are phylogenetically related and share homology in the amino acid sequence of epitopes but cross-reactivity can also involve distant species and carbohydrate determinants.[56] Therefore, we calculated the "major reactivity" for each patient and found that 60% reacted against *A. fumigatus* and *A. alternata*. Other patients (28.7%) had their major reactivity directed against the indoor species *P. chrysogenum*, *P. brevicompactum*, *C. sphaerospermum*, and *C. cladosporïoides*. These observations suggest that a genuine sensitization to indoor species can also play a role in the development of allergic asthma. Noteworthy, *A. versicolor*, albeit frequently isolated in dwellings, was never identified as "major sensitizer" in the group of asthmatics. Moreover, in mice, it failed to induce a significant IgE production.

Based on the results gathered in the murine model and in asthmatic patients, we aimed at establishing a ranking between rather inflammatory or rather allergenic mold species (Figure 10). Based on the mouse model results (early and late cytokine secretion profiles, cell recruitment in the BAL fluids and serum IgE production), species, such as *C. sphaerospermum* or *A. alternata*, seem to trigger inflammation rather than allergy. On the contrary, *P. chrysogenum* and *A. fumigatus* seem more pro-allergenic. The mixed profile of *A. versicolor* and *C. cladosporioides* was already addressed above. Of note, this classification should be considered as a continuum rather than a black or white distinction. In asthmatics, the profile is a bit different. As one could have expected from the mouse model results, *A. fumigatus* and *P. chrysogenum* emerged as strong sensitizers but *A. alternata* did so as well. This discrepancy for *A. alternata* between results obtained in the mouse model, and the sensitization of asthmatics has at least two potential explanations. Firstly, spores of *A. alternata* are much bigger (between 20 and 100 µmol/L) than spores of other species (smaller than 10 µmol/L)[57] and they may possibly interact differently with the lung tissue in mice and humans. Secondly, because of their pro-cachectic effect, the concentration of spores of *A. alternata* used for lung instillation was ten times lower than for the other species. It is therefore possible that the allergenic properties of *A. alternata* were "attenuated" in our mouse model.

Our study has several limitations. Nasal instillations of mold spores (wet aerosol) do not represent the natural lung exposure to allergens in humans. Indeed, during physiological exposure, allergens settle in limited areas of the lungs where they can trigger an inflammatory response.[18,58] After nasal instillation, such as in experimental models, allergens are widely spread in the bronchial tree and different mechanisms can be triggered explaining the apparent discrepancies. Another limitation of the study is the fact that the concentration of spores used in the mouse model that is needed to counter the lower natural

susceptibility ($10^5$-$10^6$/instillation) is much higher than spore count usually present in the air (up to 20 000 spores/m$^3$ for *Cladosporium spp*, up to 7500 spores/m$^3$ for *A. alternata* and much less for the other genus[1]) and thus largely exceed natural concentration. Responses from the mouse model may therefore differ from responses observed in the asthmatic group where individuals have an increased sensitivity to mold exposure.

In summary, results from asthmatic patients show that species with an observed low allergenic potential in mice can be linked to asthma and require attention. Results from human and murine experiments have also shown that in addition to spores of the classical outdoor species *A. alternata* and *A. fumigatus*, spores from indoor mold species can also exert strong inflammatory and allergenic properties. Therefore, considering their harmful nature, future studies should focus on investigating their role in allergic diseases.

## ACKNOWLEDGEMENTS

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