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ORIGINAL ARTICLE/ARTICLE ORIGINAL

***Aspergillus fumigatus*-sensitive IgE is associated with bronchial hypersensitivity in a murine model of neutrophilic airway inflammation**

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KEYWORDS

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Summary Neutrophils are the predominant inflammatory cells that infiltrate airways during acute exacerbation of asthma. The importance of *A. fumigatus* sensitization, and IgE response in the airways in patients with acute asthma is unclear. Rockefeller (RK) mice were sensitized with *A. fumigatus* extract protein. The animals were subsequently challenged with different degrees of *A. fumigatus* contamination in the cage bedding. All groups of mice were euthanized to obtain bronchoalveolar lavage fluid (BALF) for cytological and Elisa assays, and lung tissue for histological analysis. Moreover, several bioassays were conducted to determine whether BALF IgE antibodies can activate mast cells. In this study, we demonstrated that exposure of sensitized mice to a known concentration of *A. fumigatus* conidia produces bronchial hyperreactivity with marked neutrophilic bronchial infiltration and increased BALF IgE, capable of triggering mast cell degranulation. This study suggests that IgE may play a role in bronchial hyperreactivity associated to *A. fumigatus* exposure in mice. Mice sensitized and challenged with this fungus showed characteristics of severe asthma, with an increase of BALF neutrophils, histological changes consistent with severe asthma and an increase of IgE capable of triggering type I hypersensitivity.

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Introduction

Fungi have been associated with asthma and other airway diseases. Humans and animals inhale viable and nonviable fungi or their components in many indoor and outdoor environments, and exposure to molds can be a significant risk to human and animal health [1–3].

Aspergillus fumigatus is a saprophyte fungus, which survives and grows over a large variety of organic remains and whose most common ecological niche is on the ground. It is one of the most ubiquitous fungi, due to the ease of dispersion of its conidia [4]. However, due to their small size (2–3 µm in diameter) and hydrophobicity, the spores (conidia) may remain suspended in the air for a long time, increasing the likelihood of inhalation deep into the alveolar spaces of lungs [5,6]. Exposure to *A. fumigatus* spores is ubiquitous and symptomless for most people. However, it causes a spectrum of diseases in susceptible hosts. Invasive aspergillosis is a serious disease that may occur in immunocompromised individuals [7].

In immunocompetent patients, *A. fumigatus* can produce allergic bronchopulmonary aspergillosis (ABPA), allergic rhinosinusitis, and asthma [8–10]. *A. fumigatus* produces a significant number of allergenic molecules that react with IgE in asthmatic patients and in patients with ABPA [9]. Inhalation of *A. fumigatus* spores can lead to colonization and, in damaged airways with retained mucus, germination within the bronchial tree through the production of hyphae. In some individuals, this stimulates a T helper (Th) type 2-mediated inflammatory response involving CD4+ T cells, IgE, and IgG antibodies [11]. Moreover, in the last few years, a number of researchers have shown that B cells play a critical role in pulmonary fungal allergies [12–17].

Asthma is a heterogeneous group of conditions that result in recurrent, reversible bronchial obstruction. Although the disease can start at any age, the first symptoms occur during childhood in most cases. Asthma has a strong genetic component, and genome-wide association studies have identified variations in several genes that slightly increase the risk of disease [18]. This chronic airway disease that affects more than 300 million people is responsible for one in every 250 deaths world-wide [19]. Airway eosinophilic inflammation can cause mild to moderate asthma; however, neutrophils are crucial to airway inflammation in acute and in persistent asthma, particularly in asthma with poor response to glucocorticoid treatment [19]. The role of neutrophils in the development of bronchial asthma is complex and the functional role of these cells in disease progression remains unclear, as they can be a potent source of cytokines and inflammatory mediators participating in asthma [20,21]. Neutrophils are the predominant inflammatory cells that infiltrate airways during acute exacerbation of asthma [22]. Sensitization to *Aspergillus fumigatus* and culture in sputum samples are associated with increasing severity (particularly asthma exacerbation) of disease and airflow limitation in asthma [23–27].

The importance of *A. fumigatus* sensitization, colonization and IgE response of the airways in patients with asthma is unclear. In this study, we demonstrated that exposure of sensitized mice to a known concentration of *A. fumigatus* conidia produces bronchial hyperreactivity with marked

neutrophilic bronchial infiltration and increased BALF IgE, capable of triggering type I hypersensitivity.

Materials and methods

Animals

All of the experiments in this study were done with 5-month-old, sex- and age-matched Rockefeller (RK) mice and 3-month-old Sprague-Dawley rats. These animals were obtained from and maintained at the Animal Facility of the Universidad Austral de Chile. During exposure to *A. fumigatus*, all animals were placed in an isolation room with appropriate ventilation and filtering systems. This study was approved by the Bioethics committee for the use of animals in biomedical research of the universidad Austral de Chile.

Antigenic preparations of *A. fumigatus*

The culturing of *A. fumigatus* was carried out in two steps. The fungus was first cultured in peptone agar for 7 days at 37 °C and then in malt broth for an additional 7 days. The resulting fungi were used for soluble protein extraction and for the contamination of mouse bedding. For protein extraction, fungi were blended and subsequently sonicated (Ultrasonic Homogenizer, series 4710 Cole Parmer, USA). The resulting suspension was centrifuged at 12,500 g for 10 min and the supernatant, which contained the soluble proteins, was stored at –20 °C for later use.

Preparation and quantification of *Aspergillus fumigatus* conidia in hay

To expose mice to *A. fumigatus*, good quality hay was sterilized and sprinkled with water containing fungus conidia. The contaminated hay was then placed into plastic bags and incubated for 48 hours or more until mold contamination became visible. At this point, the contaminated hay was used as bedding in the mouse cages. Animals were maintained under these conditions according to the design of each experiment.

The quantification of *A. fumigatus* conidia in hay was performed using the method described by Araya and Zaror [28] using a Burkard manual sampler. For this, a slide covered with a drop of the following preparation was used: 5 g of gelatin, 4 g of phenol, 40 mL of glycerin, 15 mL of distilled water containing 0.01% safranin. 100 g of hay were put in a plastic bag, shaken and the sampler located in the mouth of the bag for 3 to 5 seconds. The slide was then removed and examined under a light microscope, using 100X magnification, and the samples were classified in the following categories according to Clarke and Madelin [29]: I less than 5 conidia per field; II between 5 and 10 conidia per field; and III more than 10 conidia per field 21.

Induction of neutrophilic airway inflammation

RK mice were sensitized with 50 µg *A. fumigatus* extract protein with 2 mg of aluminum hydroxide diluted

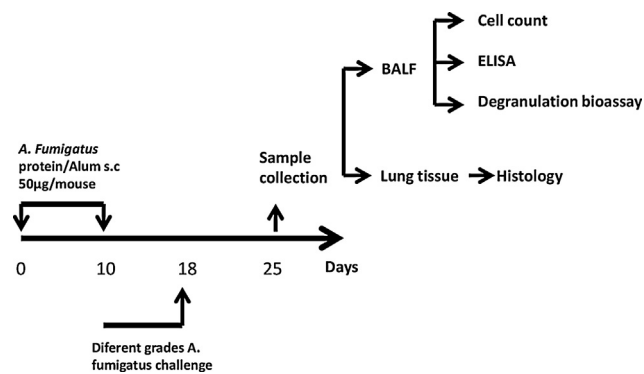


Figure 1 Schematic representation of the study. Mice were sensitized to fungal antigen by subcutaneous injection of *A. fumigatus* antigen mixed with aluminum hydroxide in PBS on days 0 and 10. Mice were then challenged with bedding with different degrees of *A. fumigatus* conidia contamination from day 18 until day 25, after which BALF and tissue samples were taken.

in phosphate buffer saline (PBS), via subcutaneous (s.c.) injection on days 0 and 10. Subsequently, the animals were challenged with different degrees of *A. fumigatus* contamination (grade 1–3) in the beds on day 18 until day 25 (Fig. 1). A control group was exposed to sterile hay. On day 25, all groups of mice were euthanized to obtain bronchoalveolar lavage fluid (BALF) and lung tissue for histological analysis.

Bronchoalveolar lavage fluid and lung histology

Bronchoalveolar lavage fluid (BALF) and lung tissues were obtained from different groups of mice, in order to prevent any possible alteration of the histological appearance of the lungs due to the lavage. The animals were euthanized using an overdose of sodium barbitol (Serve, USA). For BALF obtention, the trachea was surgically exposed and cannulated with an intravenous catheter (22 G × 1/in), and 400 µL of saline solution were slowly administered into the trachea and immediately recovered. The BALF obtained was centrifuged at 690 g. The cell pellet was used for cytological analyses by diff quick stained. For histological sections, lung tissue samples were fixed in 4% formaldehyde, embedded in Paraplast, cut into 5-µm sections, and stained with hematoxylin-eosin.

Analysis of antibody production

The levels of *Aspergillus*-specific immunoglobulins present in BALF were determined by ELISA. Briefly, 96-well polystyrene microplates (Sarstedt, USA) were coated with 10 µg/mL of *A. fumigatus* total protein dissolved in carbonate buffer (pH 9) and incubated overnight. The plates were blocked with a solution of 5% non-fat milk, followed by the addition of 100 µL of the appropriate dilution of BALF (1:1) into each well. The plates were incubated at 37 °C for 2 hours, washed three times and incubated with peroxidase-conjugated anti-mouse IgE, IgG1 or IgG2a antibodies (Santa Cruz, Inc, USA) for an additional 2 hours at 37 °C. After incubation, the plates were thoroughly washed and the reaction was revealed with o-phenylenediamine (OPD; Sigma, USA). The colorimetric

reaction was evaluated at 450 nm using a microplate reader (ELx800, Bio-Tek Instruments, USA).

Mast cell degranulation test

Fresh mast cells were obtained from 3-month-old Sprague-Dawley rats by intraperitoneal lavage with 10 mL of heparinized Hank's balanced salt solution. Cells were pooled and washed twice and resuspended at the required concentration in RPMI-1640 medium containing 10% fetal calf serum (FCS). For degranulation tests, rat peritoneal mast cells were separated by glass adherence on covered slides and were adjusted to a concentration of 10^6 cells/mL in RPMI-1640 medium containing 10% FCS plus antibiotics as previously described [30,31]. Later, the cells were incubated for 2 h at 37 °C in the presence of 100 µL of BALF obtained from the different groups of mice. Further, BALF was subjected to a thermal shock at 56 °C (30 min) to eliminate other subclasses of immunoglobulins (IgG) capable of stimulating mast cells, thus retaining only BALF IgE [23]. Cells incubated without BALF were used as the negative control. Each group received 77 µg of soluble protein obtained from *A. fumigatus* via incubation at 37 °C for 15 min [23]. PBS solution was used as the incubation control. Total mast cell degranulation induced with 0.5 µM digitonin (Sigma Chemical Co, USA) was used as a positive control. Finally, all cells were fixed with 10% formalin and absolute ethanol and stained with 0.1% toluidine blue in order to determine mast cell degranulation (percentage). Two hundred or more mast cells were counted per group. The extent of degranulation was determined as previously described [30,31]. All determinations were carried out in duplicate, and results are shown as the means of three experiments with different pools of mast cells.

Statistical analysis

The results of BALF neutrophil content, antibody levels and mast cell degranulation were assessed by ANOVA. When significant, means were compared using Tukey's multiple comparison test. The results are reported as mean ± SD. A value of $P < 0.05$ was considered significant.

Results

Analysis of neutrophil content of BALF

The differential count of BALF cells from each study group differed according to the degree of exposure to *A. fumigatus* conidia. As shown in Fig. 2, the group of mice exposed to *A. fumigatus* grade III contamination showed a significantly higher amount of BALF neutrophils compared to both grade I, II and clean hay control groups. BALF content in the groups of mice exposed to grade I and II contaminated hay did not vary significantly from the control group.

Antibodies levels in BALF

A. fumigatus-specific immunoglobulin levels are shown in Fig. 3. A significant increase of specific IgE (Fig. 3A) was observed in the group of mice exposed to grade III *A. fumigatus* contamination, but no significant differences

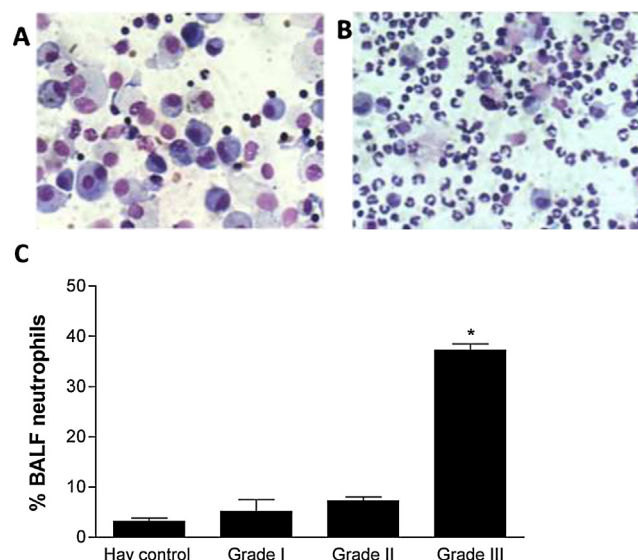


Figure 2 Bronchoalveolar fluid neutrophil content in mice after exposure to hay with different degrees of contamination with *A. fumigatus* conidia. A. Representative image of BALF froth from mice in the negative control group or mice exposed to hay with grade I or II *A. fumigatus* contamination (note the scarcity of neutrophils, less than 7%). B. Representative image of BALF froth from mice exposed to hay with grade III *A. fumigatus* contamination (note the abundance of neutrophils, approximately 40%). C. Each bar represents the mean $\pm$ SD of the relative neutrophil content (%) in BALF ($n = 8$ per group); $^*(P < 0.01)$.

were found in the other groups of animals. This same phenomenon was observed for specific Ig2a (Fig. 3B), which increased significantly in the group of mice exposed to grade III *A. fumigatus* contamination.

Mast cell degranulation bioassay

A clear increase in the relative degranulation (%) of rat peritoneal mast cells was observed after incubation with BALF from mice exposed to hay with grade III *A. fumigatus* contamination (Fig. 4A). In contrast, degranulation of rat peritoneal mast cells was not observed when these cells were incubated with BALF from mice in the negative control group (exposed to uncontaminated hay) or mice exposed to hay with grade I or II *A. fumigatus* contamination (Fig. 4B). Relative degranulation (%) of rat peritoneal mast cells in response to incubation with mice BALF was significantly higher in the group of mice exposed to grade III contaminated hay ($P < 0.001$) than in the negative control group or the groups of mice exposed to grade I and II contaminated hay (Fig. 4C).

Histopathological evaluation

Fig. 5 is a representative image of a normal bronchus of the control group of mice exposed to uncontaminated hay. The luminal surface epithelium in the bronchiolar airways of unaffected mice was composed of thin cuboidal epithelial cells. These cells were mostly non-ciliated clara cells and

ciliated cells with a few interspersed mucus cells. Fig. 5A is a representative image of a bronchus of sensitized mice exposed to grade II *Aspergillus* contamination, which shows a moderate inflammatory reaction in the terminal bronchiole and alveoli, characterized by neutrophil polymorphonuclear (PMNN) infiltration, macrophages and a smaller amount of lymphocytes. In the bronchiolar lumen, there are few PMNNs and cellular debris.

In mice exposed to hay with grade III *Aspergillus* contamination, significant bronchiolitis was observed, characterized by peribronchiolar and perivascular mononuclear and neutrophilic infiltration, accumulation of intraluminal bronchiolar mucus and serofibrinous exudates. Extensive folding of the airway epithelia was also visible, which is a sign of airway smooth muscle contraction and smooth muscle hypertrophy. The bronchiolar lumen was reduced in size due to the presence of inflammatory exudate and bronchoconstriction. Further, moderate mucosal associated lymphoid tissue hyperplasia was evident. This inflammatory response was observed predominantly in the peribronchiolar interstitial tissues and often extended into the lamina propria and luminal surface epithelium of the affected airways. We also observed infiltration of inflammatory cells into the alveolar space.

Discussion

In previous studies, both invasive and non-invasive methods have been employed to understand the nature of host-*aspergillus* interaction in the genesis of *A. fumigatus*-induced pulmonary disease [32–35]. In this study, animals were challenged with a known amount of *A. fumigatus* conidia in the animals' bedding, which was changed daily to maintain a steady level of exposure. This non-invasive method allows repeated exposure to the antigen, as occurs in humans, resulting in constant conidia dissemination through the lungs. We showed that severe inhalation (grade III) of *A. fumigatus* conidia elicited significantly pulmonary inflammation, bronchial antibody production and histopathological changes associated to bronchial hyperreactivity, which suggest that constant inhalation of *A. fumigatus* conidia may elicit symptoms of neutrophilic asthma.

Severe asthma is often characterized by neutrophilic inflammation, either in the presence or absence of Th2-induced eosinophilic inflammation [36,37]. *A. fumigatus* detection in sputum is associated with *A. fumigatus*-IgE sensitization, neutrophilic airway inflammation, and reduced lung function [38]. This supports the concept that development of fixed airflow obstruction in asthma is a consequence of the damaging effects of airway colonization with *A. fumigatus* [35]. In this work, we demonstrated that mice which were sensitized and exposed to spores of *A. fumigatus* produced an increase of IgE antibodies, which was associated with bronchial hyperreactivity and an increase of BALF neutrophils. In addition, these IgE antibodies had the ability to induce mastocyte degranulation (from non-sensitized rats) in response to exposure to *A. fumigatus*.

IgE is greatly responsible for mediating hypersensitivity; however, other subclasses of IgG have been proposed to mediate mast cell degranulation in humans [39,40], and

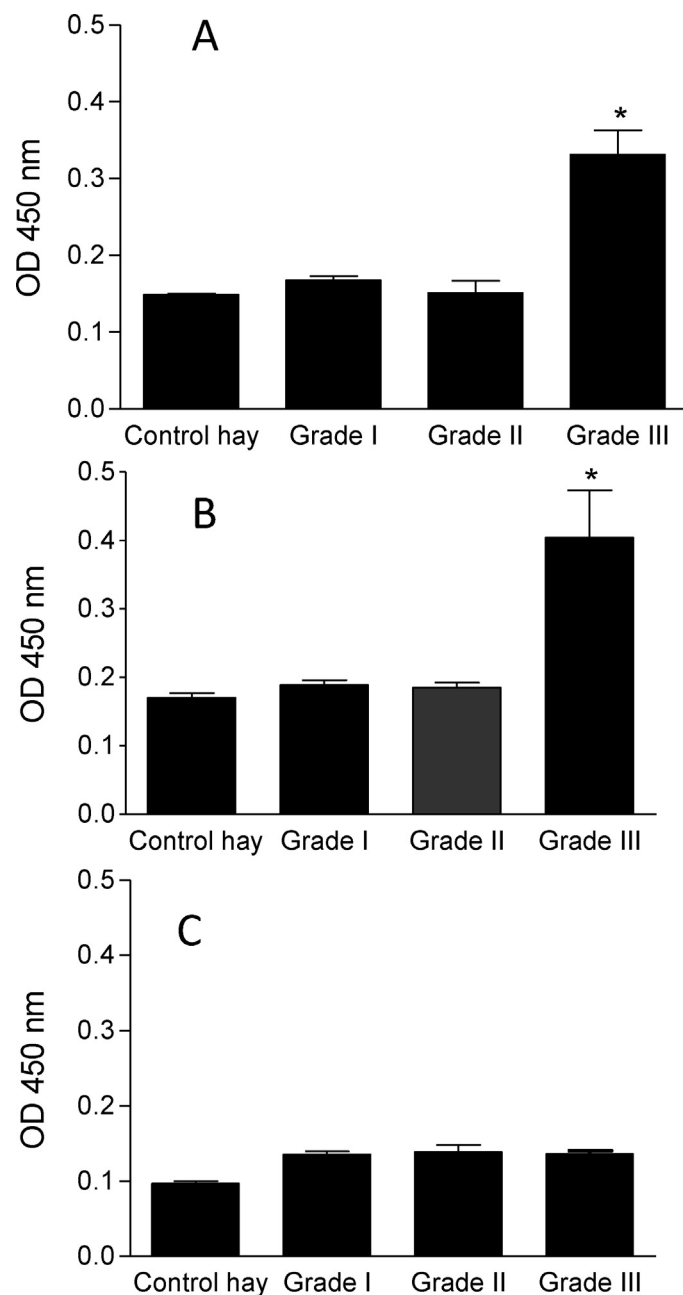


Figure 3 Specific IgE (A), IgG2a (B) and IgG1 (C) levels against *Aspergillus fumigatus* in BALF (1:1) in mice after exposure to hay with different degrees of contamination with *A. fumigatus* conidia. Each bar represents the mean of the optical density (OD) of each group ($n = 8$ per group); * ($P < 0.05$).

mice [41,42]. Our experiments cannot determine which immunoglobulin isotype mediated mast cell activation. However, mouse IgE antibodies are partially protected against heat inactivation at 56 °C when the sera are diluted in saline or phosphate media [31,43] and this characteristic is generally absent in the IgG subclasses. Based on these facts, we hypothesize that IgE immunoglobulins play important roles in these *in vitro* bioassays. Since the bioassay used in this study allowed detection of *A. fumigatus*-specific BALF IgE (Fig. 3), our results suggest that IgE could effectively be involved in the immunological mechanisms leading to the development of neutrophilic asthma associated to *A. fumigatus* sensitization.

Neutrophils are the predominant inflammatory cells that infiltrate airways during acute exacerbation of asthma [22]. Further, neutrophils also play an essential role in antifungal defense [44,45]. They rapidly migrate to the infected lungs and suppress conidial germination through phagocytosis or extracellular trap formation [46,47]. Other authors demonstrated that experimental exposure of mice to *A. fumigatus* conidia induced an increase in the ingestion potential and number of conducting airway neutrophils [48]. The same authors suggested that this priming of neutrophils is sufficient to control the release of the non-germinal conidia of *A. fumigatus*, and could partly explain the development of sensitization to fungi and subsequent bouts of exacerbated

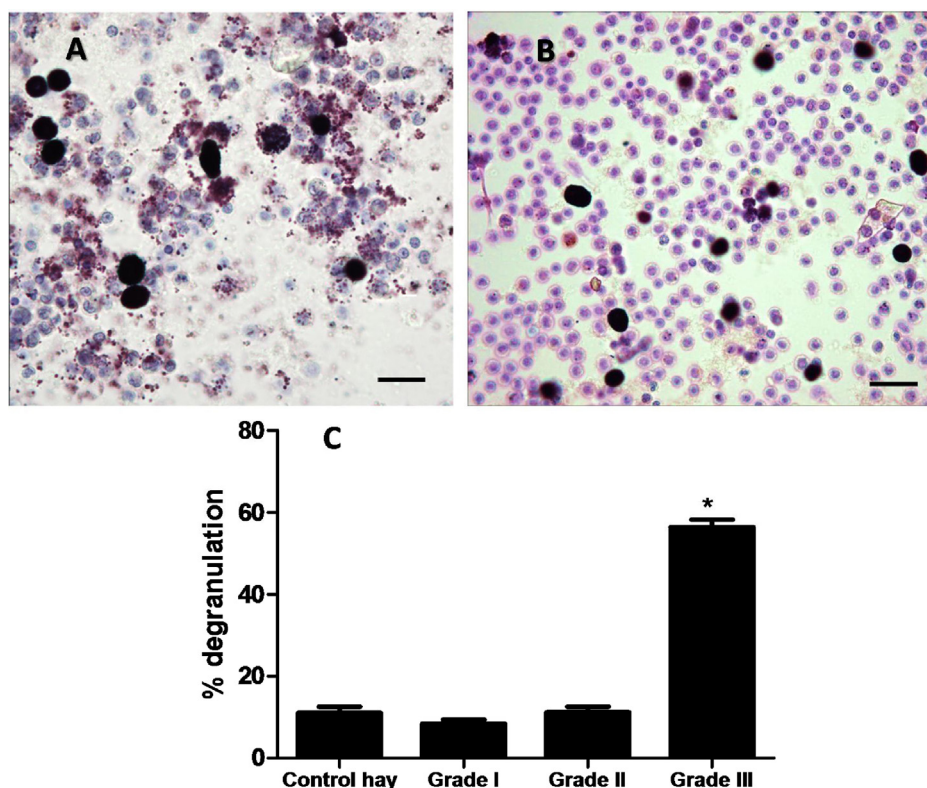


Figure 4 Rat peritoneal mast cell degranulation bioassay. A. Representative image of rat peritoneal mast cells with positive degranulation, after incubation with BALF from mice exposed to hay with grade III *A. fumigatus* contamination. B. Representative image of rat peritoneal mast cells with negative degranulation, after incubation with BALF from mice in the negative control group or mice exposed to hay with grade I or II *A. fumigatus* contamination; scale: 20 μm. C). Rat peritoneal mast cell degranulation (%) in response to incubation with BALF from mice exposed to control hay (uncontaminated) and hay with increasing grades of *A. fumigatus* conidia contamination (* $P < 0.01$).

asthma upon exposure to known pathogens. Our results agree with this line of thought, since mice sensitized and severely exposed to *A. fumigatus* conidia (grade III) showed marked bronchial hyperreactivity, which suggests that the extent of airway inflammation is directly related to the amount of conidia in the environment.

Our results show that the immune response against *A. fumigatus* spores, in sensitized mice, is characterized by increased levels of antigen-specific IgE and IgG2a antibodies in the BALF. Pandey et al. [35] suggest that these immunoglobulins may play a critical role in host defense against respiratory pathogens and inhaled allergens. The increased BALF IgE levels suggest that a type I hypersensitivity response may be involved in the pathogenesis of *A. fumigatus*. However, the increase in BALF IgG2a levels in mice that were exposed to the allergen is also compatible with a Th1 response [49]. Moreover, IgG2a can activate the complement cascade, which may partly explain the increase in neutrophils observed in the BALF of mice that were exposed to *A. fumigatus*. Our results are in concordance with studies in human ABPA patients [50] and mice [35], in whom elevated BALF IgE and IgG levels have been detected. All of the above suggests that inhalation of *A. fumigatus* conidia might play an important part in the development of allergic airway disease. Studies that used IL-4, IFN- γ and IL-12 knock-out mice showed that these cytokines each play an important role in the inflammatory process [51]. However,

each of these cytokines differentially contributes to the inflammatory process and their individual contributions are based on the route of immunization [51]. In addition, some groups reported different outcomes of the inflammatory process depending on whether the antigen was delivered orally or nasally: according to the route of administration, the resulting inflammation mimicked either eosinophil- or neutrophil-mediated asthma-like diseases [52]. Here, the challenge of mice with *A. fumigatus*-contaminated bedding resulted in a significant increase in BALF neutrophils, changes in lung histology and rat mast cell degranulation.

Resident lung cells have also been implicated in the onset of the airway inflammatory response against *A. fumigatus*. Many resident lung cells express lectin and toll-like receptors that can interact with *Aspergillus* components. Thus, inhalation of the fungus alone may induce the production of proinflammatory, Th-1 and Th-2 cytokines, which may be sufficient to trigger the onset of respiratory airway inflammation [53,54]. In our study, progressive fungus aspiration generated a progressive and specific immune response; this response is probably modulated by cytokines from the innate immune system. In this sense, other results from our laboratory indicate that SAA protein (acute phase protein) levels increased in both serum and lung within 2–24 hours after mice were exposed to *Aspergillus* spores. SAA mRNA expression levels in the lungs and BALF cells also increased within the first hour after mice were exposed to *A. fumigatus* [55].

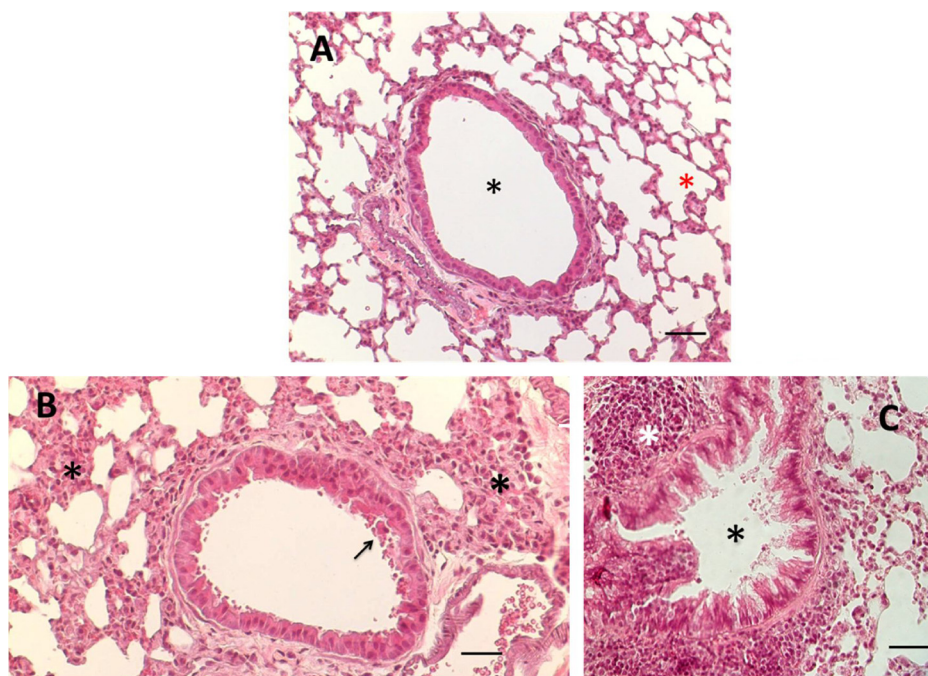


Figure 5 Fig. 5A is a representative image of a normal bronchus of the control group of mice exposed to uncontaminated hay. The luminal surface epithelium in the bronchiolar airways of unaffected mice was composed of thin cuboidal epithelial cells. These cells were mostly non-ciliated clara cells and 260 ciliated cells with a few interspersed mucus cells. Fig. 5B is a representative image of a bronchus of sensitized mice exposed to grade II *Aspergillus* contamination, which shows a moderate inflammatory reaction in the terminal bronchiolus and alveoli, characterized by neutrophil polymorphonuclear (PMNN) infiltration, macrophages and a smaller amount of lymphocytes. In the bronchiolar lumen, there are few and cellular debris. In mice exposed to hay with grade III *Aspergillus* contamination Fig 5C, significant bronchiolitis was observed, characterized by peribronchiolar and perivascular mononuclear and neutrophilic infiltration, accumulation of intraluminal bronchiolar mucus and serofibrinous exudates. Extensive folding of the airway epithelia was also visible, which is a sign of airway smooth muscle contraction and smooth muscle hypertrophy. The bronchiolar lumen was reduced in size due to the presence of inflammatory exudate and bronchoconstriction. Further, moderate mucosal associated lymphoid tissue hyperplasia was evident. This inflammatory response was observed predominantly in the peribronchiolar interstitial tissues and often extended into the lamina propria and luminal surface epithelium of the affected airways. We also observed infiltration of inflammatory cells into the alveolar space.

Those results proved that *A. fumigatus* induced an acute inflammatory response in the airways through the stimulation of SAA protein, which increased in serum, lung tissue and BALF samples during the early hours of fungal exposure in previously sensitized mice. Thus, innate immunity components seem to play a pivotal role in the first hours of fungal exposure in *A. fumigatus*-sensitive mice.

In conclusion, this study proves the participation of IgE in the bronchial hyperreactivity associated to *A. fumigatus* exposure in previously sensitized mice. These mice showed characteristics of severe asthma, with an increase of BALF neutrophils, histological changes consistent with severe asthma and an increase of IgE capable of triggering type I hypersensitivity.

Disclosure of interest

The authors have not supplied their declaration of competing interest.

Acknowledgments

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