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Age influence on mice lung tissue response to *Aspergillus fumigatus* chronic exposure

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Abstract

Introduction and objective. Exposure to conidia of *Aspergillus fumigatus* was described as a causative factor of a number of the respiratory system diseases, including asthma, chronic eosinophilic pneumonia, hypersensitivity pneumonitis and bronchopulmonary aspergillosis. The study investigates the effects of the repeated exposure to *A. fumigatus* in mice pulmonary compartment. Our work tackles two, so far insufficiently addressed, important aspects of interaction between affected organism and *A. fumigatus*: 1) recurrent character of exposure (characteristic for pathomechanism of the abovementioned disease states) and 2) impact of aging, potentially important for the differentiation response to an antigen.

Materials and methods. In order to dissect alterations of the immune system involved with both aging and chronic exposure to *A. fumigatus*, we used 3- and 18-month-old C57BL/6J mice exposed to repeated *A. fumigatus* inhalations for 7 and 28 days. Changes in lung tissue were monitored by histological and biochemical evaluation. Concentration of pro- and anti-inflammatory cytokines in lung homogenates was assessed by ELISA tests.

Results and conclusions. Our study demonstrated that chronic inflammation in pulmonary compartment, characterized by the significant increase of proinflammatory cytokines (IL1, IL6, IL10) levels, was the dominant feature of mice response to repeated *A. fumigatus* inhalations. The pattern of cytokines' profile in the course of exposure was similar in both age groups, however in old mice the growth of the cytokines' levels was more pronounced (especially in case of IL1).

Key words

Aspergillus fumigatus, inflamm-aging, cytokines' profile, chronic exposure, mouse model

INTRODUCTION

Fungi of the genus *Aspergillus* often cause exacerbations of asthma and other respiratory allergic diseases [1, 2]. The species *Aspergillus fumigatus* is responsible for over 80% of *Aspergillus*-related pulmonary disorders, including asthma, chronic eosinophilic pneumonia, hypersensitivity pneumonitis and bronchopulmonary aspergillosis [3]. This fungus may act as an allergic, toxic or infectious agent.

Exposure to *A. fumigatus* occurs via inhalation of intact conidia and fragments of mycelium and conidia, which can pass through the upper respiratory tract and reach the distal airways, terminal bronchi and pulmonary alveoli. The immune response to inhaled *A. fumigatus* is characterized by interaction between innate and adaptive immunity, both of which are activated on exposure to the fungus. Host defence against primary *A. fumigatus* infection is mediated by phagocytic cells. Alveolar macrophages devour and kill inhaled *Aspergillus* conidia, while neutrophils destroy hyphae that sprout from conidia not eliminated by macrophages [3, 4, 5, 6, 7]. Macrophages, not only phagocyte fungus, following recognition, express a wide variety of cytokines and chemokines critical for prevention of *A. fumigatus* invasion and destruction of pulmonary tissue [8, 9, 10, 11, 12]. Furthermore, macrophages play an important role in: 1) regulating the balance between the proinflammatory and anti-inflammatory cytokine responses that are required for recruitment and activation of neutrophils, and 2) in augmenting or attenuating cellular immunity [13]. Innate immunity is able to control *A. fumigatus* infection, especially at lower doses, but when the infections become frequent or high fungal burdens occur, adaptive immunity is necessary to provide a sufficient level of protection [14]. Different types of the immune system's responses were observed depending on the fungal specific components to which T lymphocytes were exposed. Fungal proteins and glycolipids activated nonprotective (Th2) IL4 or (Th17) IL17 secreting clones; on the contrary, polysaccharides induced protective IFNγ, IL17, or IL10-clones leading to Th1/Treg protective reactions [15].

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Typically, *A. fumigatus* conidia exert their pathological activity after being repeatedly inhaled into airways [16]. Despite this fact, most studies have focused on the immune response following one or a limited number of exposures to *A. fumigatus* conidia [17, 18, 19, 20, 21]. Therefore, little is known about the regulation of the host response to repeated exposure to *A. fumigatus* conidia. The authors of the presented study have previously reported that chronic exposures of 3-month-old C57BL/6J mice to saline extract of *A. fumigatus* mycelium containing conidia result in pulmonary inflammation [22]. A key limitation of above-mentioned studies was lack of analysis of *A. fumigatus* influence on the mice immune system.

Furthermore, the design of the presented study allows analysis of the impact of age at the time of infection. Aging is accompanied by quantitative and qualitative changes in the immune system, resulting in an increased susceptibility to neoplasias, infections and autoimmune disorders [23, 24, 25, 26]. It has been suggested that one of the major characteristics of aging is the pro-inflammatory environment [27, 28]. However, studies examining alterations in pro-inflammatory cytokine production in the elderly have brought contradictory results. Although several studies have shown a decrease in the production of pro-inflammatory cytokines by macrophages from aged humans and mice, the opposite changes have also been reported [29, 30, 31]. The ageing process has an impact not only on macrophages but also influences the function of T cells [32, 33, 34]. Aging differentially affects the Th1 and Th2 subsets, although here the data is again ambiguous [27, 35, 36, 37]. Some studies revealed that a shift towards an increased role of Th2 cytokines and a diminished role of Th1 cytokines emerges with aging [27, 36, 37]. Other studies suggest that the microenvironment in which CD4+ T cells develop in older people may cause the production of more Th1 cells, compared to younger individuals [35, 38, 39]. Nevertheless, most of studies revealed that aging is associated with an increase in pro-inflammatory cytokines (IL1, IL6, TNFα, IFNγ), which is accompanied with stimulation of release of anti-inflammatory mediators (IL10, TGFβ) [40, 41, 42, 43, 44, 45, 46]. Thus, these cytokines could be described as markers of aging.

In order to segregate alterations of the immune system involved with aging from those connected to chronic exposure to *A. fumigatus*, the presented study quantifies the cytokines levels in mice lung tissue homogenates, and records the histological changes in lung tissue in the course of repeated inhalations of harmful fungus.

OBJECTIVES

The aims of the study were:
1) to analyze the immune response in the pulmonary compartment to repeated *A. fumigatus* exposure;
2) to demonstrate whether age, and especially the 'inflamm-aging' phenomenon, influences these immune reactions.

MATERIALS AND METHODS

Animals. 3-month-old and 18-month-old female C57BL/6J mice were purchased from Charles River Laboratories, GmbH, Germany. The mice were fed a standard diet and water *ad libitum,* housed under standard conditions. The experimental protocol was approved by the Local Bioethics Committee in Lublin, Poland.

Saline extract of *Aspergillus fumigatus* (SE-AF). A standard strain IG-1 obtained from the Institute of Tuberculosis and Pulmonary Diseases in Warsaw was inoculated on nutrient broth (BTL) supplemented with 4% glucose. Cultures had been incubated in Erlenmayer flasks for 21 days at 30°C. Superficial mycelium was suspended in culture broth and homogenized with a laboratory mixer. Next, the cell suspension was extracted in saline (0.85% NaCl) in the proportion 1:2 for 48 hrs at 4°C, with intermittent disruption of cells by 10-fold freezing and thawing. Afterwards, the supernatant was separated by centrifugation, dialysed against tap water for 48 hrs, and then against distilled water for 24 hrs, and finally lyophilized.

Mice exposure to saline extract of *Aspergillus fumigatus*. C57BL/6J mice were exposed for 28 days, one hour daily, to a finely dispersed aerosol of the saline extract of *Aspergillus fumigatus* mycelium (SE-AF). The extract was dissolved in saline in the concentration of 1 mg/ml. Animals' exposure was performed using the novel inhalation challenge set constructed according to own design [47], consisting of an ultrasonic aerosol generator, an airtight chamber with 15 perforated containers for mice, and a vacuum pump. This set assured the continuous flow of a fine droplet aerosol composed of fine particles (fragments of conidia and mycelium), measuring on average 1.77 μm, which could easily penetrate into the deep parts of the lungs, alveoli and bronchioli. The intact conidia, measuring on the average 2.1 μm, were deposited mainly in the terminal bronchi. Concentration of the aerosolized extract in the chamber, measured by weighing filters mounted at the outlet of chamber, both before and after exposure, was in the range (x ± S.D): 67.6 ± 31.3 – 112.8 ± 50.3 μg/m^3.

The lung samples were taken before treatment and after 7 and 28 days of mice exposure to SE-AF. In young mice, samples were collected from 4, 6, and 6 mice, respectively. In the case of old mice, samples were collected from 3 individuals at each of time points.

Histological examination – hematoxylin and eosin staining. Lung tissue was cut into pieces 2 ´ 5 mm thick and fixed in 4% buffered formalin for 12 hrs, followed by dehydration in 95% etanol, and further dehydrated in ascending series of alcohol, and embedded in paraffin wax. 5 μm thick sections were obtained from paraffin blocks and stained with haematoxylin and eosin.

After haematoxylin-eosin staining, the effect of *A. fumigatus* exposure on mice lung morphology was assessed using light microscopy. Histological examination was performed by a pathologist who was blinded to the experimental protocol; the images were obtained using an image analysis system (D-Sight system, Menarini Diagnostics, Florence, Italy). Lung injury was scored according to the following 4 features: centrolobular inflammation, interstitial inflammation, peribronchial fibrosis and interstitial fibrosis. These features were graded on a 4-point scale: 0 = regular tissue, 1 = mild changes, 2 = moderate changes, 3 = significant changes. Individual parameters: lung inflammation score and lung fibrosis score, were calculated separately as the mean of investigated items in each research group.

Biochemical examination – hydroxyproline level determination. Lavaged lungs were perfused via the right heart ventricle with saline, excised and placed in a Falcon tube chilled on ice. 2 ml of 0.9% saline were added and the lungs were then homogenized with an Ultra-Turrax T25 homogenizer (Janke and Kunkel, Brussels, Belgium) for 30 seconds and stored at -80 °C for later use. Collagen deposition was estimated by measuring the hydroxyproline contents of lung homogenates by high-performance liquid chromatography (HPLC), as previously described [48].

Immunological examination – determination of cytokines concentrations. Lung tissue was homogenized mechanically in the presence of protease inhibitors. After centrifugation, obtained homogenates were stored at -20 °C for later use.

Concentrations of TNFα, TGFβ1, IL1, IL6, IL10 and IFN-γ, in lung tissue homogenates were measured by cytokine specific ELISA kits according to manufacturer's instructions. The following kits (Gen-Probe Diaclone, Besancon Cedex, France) were used: Murine TNF-α ELISA Kit, Mouse TGF-β1 ELISA Kit, Murine IL-1 ELISA Kit, Murine IL-6 ELISA Kit, Murine IL-10 ELISA Kit, Murine IFN- γ ELISA Kit.

Statistical analysis. Statistical analyses were performed by GraphPad Prism 5. Data obtained from histological and immunological examination were presented as the mean value and standard error of the mean (SEM). Data obtained from hydroxyproline assay were presented as the median value, 25 – 75% percentile and range. For assessment of histological, immunological and biochemical data, statistical analysis was performed using the t-test. Significance was accepted at $p<0.05$.

RESULTS

Histological evaluation – H&E staining. Results of histological examination showed that after 7 days of exposure to SE-AF young mice revealed a low-grade of inflammation (Fig. 1, Fig. 2C, mean score 0.667 according to the criteria described in the Methods). The inflammation process progressed after subsequent days of treatment (Fig. 1, Fig. 2E, mean score 1.583). Old mice manifested some inflammatory reaction, even in absence of a harmful agent. Similar to young animals, old mice revealed advancement of inflammation during inhalation. Mean score increased from 0.375 (control), through 0.750 after 7 days of inhalation to 1.400 at the end of experiment (Fig. 1, Fig. 2D and 2F). Chronic exposure of animals to SE-AF increased the level of collagen concentration in murine lungs. Observed changes were dependent on time of exposure and age of the mice. The most significant changes were noted in old mice after 28 days of inhalation with SE-AF, where the mean fibrosis score reached 0.900 (Fig. 1, Fig. 2F). Furthermore, the elevated level of collagen was also shown in untreated mice (Fig. 1, Fig. 2B, mean score 0.500). Moreover, the changes (both immune response and 'signs of fibrosis') observed in old animals in subsequent time points were not as dynamic as those observed in young animals.

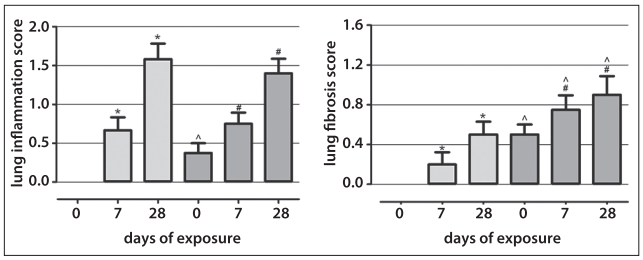

Figure 1. Quantification of inflammation and fibrosis in lung samples collected from untreated mice (control) and mice exposed to saline extract of *Aspergillus fumigatus* mycelium. Data for histologic scores are given as mean ± SEM of investigated items. * $p<0.05$ vs. control (young mice), # $p<0.05$ vs. control (old mice), ^ $p<0.05$ vs. corresponding time points in young mice. T-test. Light bars – young mice, dark bars – old mice

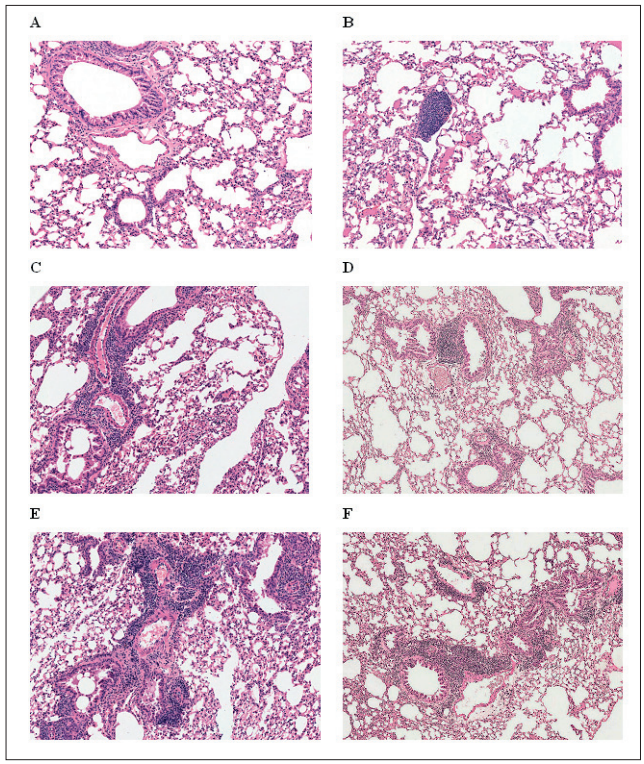

Figure 2. Histological examination of haematoxylin and eosin staining of lungs collected from untreated mice (A, B) and mice exposed to saline extract of *Aspergillus fumigatus* mycelium (C, D, E, F). Photographs C and D present the results after 7 days of inhalation; photographs E and F – after 28 days of inhalation. On the left – images derived from 3-month-old mice; on the right – 18-month-old animals

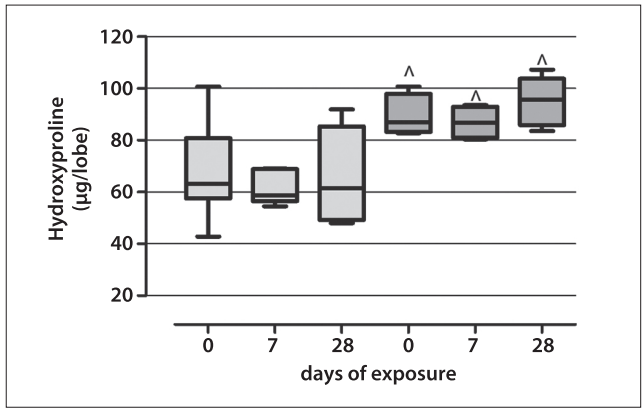

Figure 3. Hydroxyproline concentration in lung homogenates received from untreated mice (control) and mice exposed to saline extract of *Aspergillus fumigatus* mycelium. Data are given as mediana, 25–75% percentile and range (0–100% percentile).
* – $p<0.05$ vs. control (young mice);
# – $p<0.05$ vs. control (old mice);
^ – $p<0.05$ vs. corresponding time points in young mice.
T-test. Light boxes – young mice, dark boxes – old mice

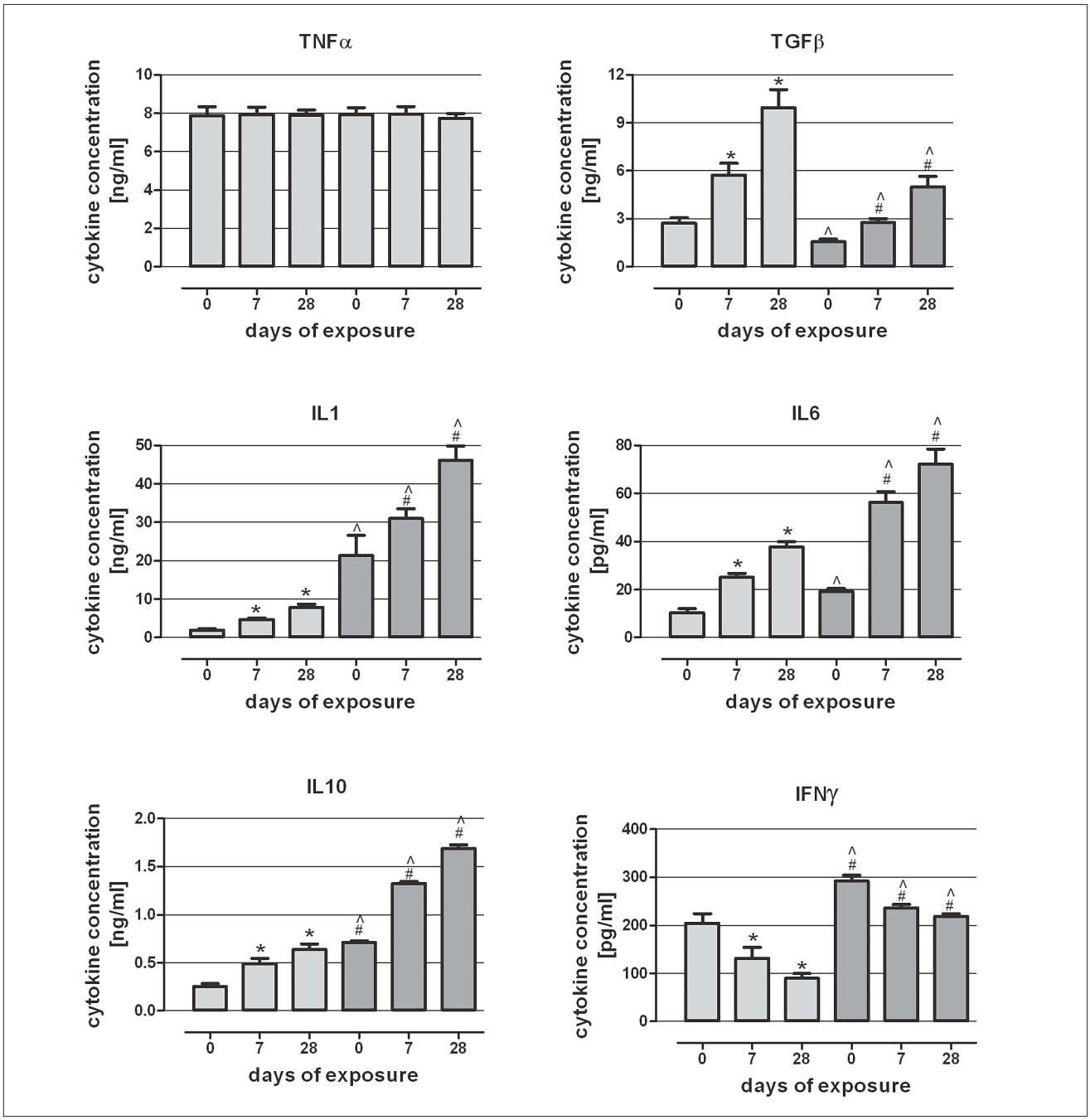

Figure 4. Cytokines' concentration in lung homogenates received from untreated mice (control) and mice exposed to saline extract of *Aspergillus fumigatus* mycelium. Data are given as mean of investigated cytokine' concentration ± SEM.
* – p<0.05 vs. control (young mice);
# – p<0.05 vs. control (old mice);
^ – p<0.05 vs corresponding time points in young mice.
T-test. Light bars – young mice, dark bars – old mice

Determination of hydroxyproline level. Amplitude of the pulmonary fibrosis induced by chronic exposure to SE-AF was determined by measuring the hydroxyproline level in lung homogenates of mice (Fig. 3). Neither young nor old mice exposed to SE-AF revealed statistically significant differences in hydroxyproline concentration, compared to untreated mice. On the contrary, a meaningful increase of hydroxyproline level was detected in old animals, i.e. the hydroxyproline concentration was more than 50% higher, compared to young mice. Obtained results corresponded with histological examination.

Changes in cytokine concentration after animals' exposure to *A. fumigatus*. Concentrations of proinflammatory cytokines (TNFα, IFNγ, IL1, IL6) and anti-inflammatory cytokines (IL10, TGFβ) are presented in Fig. 4. A significant increase in interleukins and TGFβ concentrations was noted in all mice after exposure to *A. fumigatus*. Observed changes were dependent on animals' age and time of exposure. Nevertheless, a positive correlation between mice age and cytokines level was only noted in the case of interleukines. On the contrary, IFNγ concentration decreased during mice exposure to *A. fumigatus* in both age groups; however, the

strongest inhibition was observed in 3-month-old animals. Expression of TNFα was not affected by exposure to *A. fumigatus* or by the age of the animals.

DISCUSSION

Aging is associated with a decline in a wide spectrum of immune functions. As a part of the age-associated immune system decline, chronic inflammation occurs even in the absence of a harmful agent or apparent disease [49, 50, 51]. This state, known as 'inflamm-aging', is characterized by an increased level of proinflammatory factors (IL1, IL6, TNF*α*) and anti-inflammatory mediators (IL-10, TGFβ). This phenomenon contributes to the decreased ability of an immune system to respond adequately to an infectious challenge [42, 44, 45, 46]. All the above-mentioned cytokines (potential markers of aging) are classified into monokines; however, in spite of this definition, they are also released by lymphocytes. Considering this fact and the importance of alveolar macrophages and lymphocytes for combating *A. fumigatus* load, the presented study focused on evaluation of the influence of chronic exposure to this fungus on lung tissue response, with particular reference to monokines concentration. The obtained results revealed the presence of the 'inflamm-aging' state: the concentration of IL1, IL6, IL10 and IFNγ in untreated mice was positively correlated with age. Furthermore, histological examination also revealed slight signs of inflammation in old animals not exposed to *A. fumigatus*. The concentration of IL1, IL6 and IL10 increased with exposure time in an age dependent manner, while TGFβ concentration, which increased with the time of exposure, was significantly higher in young animals. On the contrary, IFNγ concentration decreased during inhalation in both age groups; however, its level was initially much higher in old mice (although it declined at a more pronounced rate in old animals). TNF*α* concentration was not impacted by either inhalations nor by the animals' age. Thus, 4 distinct patterns in cytokine behaviour during the experiment could be distinguished:

1) increase with exposure and age (IL1, IL6, IL10);
2) increase with exposure, decrease with age (TGFβ);
3) decrease with exposure, increase with age (IFNγ);
4) no change (TNF*α*).

Previously, a number of studies demonstrated that Th cytokines contribute to phagocytic cell-mediated host defence against *A. fumigatus* load. Stimulation of cultured phagocytic cells with Th1 cytokines, including IFNγ, enhanced fungicidal activity. On the contrary, stimulation with Th2 cytokines, including IL4, IL6 and IL10, had the opposite effect [7, 52, 53, 54, 55]. *In vivo* studies confirmed that mice resistance to experimentally-induced *A. fumigatus* infections was correlated with the induction of cytokines, including TNFα, IL12, and IFNγ, while susceptibility to infection was associated with the production of IL4, IL10 [56, 57].

The significant up-regulation of IL10 concentration and decline of IFNγ, as well as the constant level of TNF*α*, revealed in the presented study are in line with the above-mentioned results, especially with the observations made by Nagai *et al.*, who observed increased IL10 expression following IFNγ neutralization, which worsened tissue pathology [57].

RESULTS

Histological examination have shown that young and old mice after 7 days of exposure to SE-AF revealed a low-grade of inflammation, the range of which was extended after subsequent inhalations. Additionally, chronic exposure to SE-AF increased collagen level in a time- and age-dependent manner (as assessed by histological analyses). It should be emphasized that the absolute magnitude of changes induced in old mice by SE-AF inhalation was similar (inflammation score) or higher (fibrosis score) compared with young mice. Nevertheless, when considering the presence of inflammatory response and 'signs of fibrosis' also in untreated old mice, the changes observed in this age group exposed to SE-AF must be recognized as not as dynamically developing as thosee noted in young animals.

In relation to cytokine profile, the most pronounced difference observed as an effect of age was the overproduction of IL1. The levels of this interleukine were increased 23-fold in old mice at the final stage of the experiment, compared to untreated young mice. IL1 is the main regulator of immune and inflammatory response, affecting almost all cell types, including macrophages and lymphocytes [58]. The level of the second cytokine also significantly different in both age groups in the course of the study IL6. A 7-fold increase in IL6 was noted in old animals after 28 days of inhalations, compared to untreated young mice. IL6, secreted by T cells and macrophages, similarly to IL1, stimulates immune response leading to inflammation. Simultaneously, this interleukine also plays a role in fighting infections [59]. IL1, together with its co-player IL6, may suppress the profibrotic action of IL10 and TGFβ, which could explain the lack of prominent fibrosis in mice treated with SE-AF. This hypothesis is particularly plausible in the case of old animals where the absolute value of IL1 was several times higher than the concentration of IL10 and TGFβ. Looking at cytokines' profile induced by *A. fumigatus*, stimulation of cytokines, which are classified to Th2 (IL6, IL10) and Treg (TGFβ) response, was seen, accompanied by a decline in IFNγ (Th1 cytokine). All cytokines in which increased levels were noted, are typical for activated macrophages (IL1, IL6, IL10, TGFβ). These observations may suggest that mice chronic exposure to SE-AF mainly involved macrophages, and shifted the immune response towards a Th2 reaction.

CONCLUSIONS

The presented study has demonstrated that chronic inflammation in the pulmonary compartment, characterized by a significant increase in pro-inflammatory cytokins (IL1, IL6, IL10) levels, was the dominant feature of mice response to repeated *A. fumigatus* inhalations. The pattern of cytokines' profile in the course of exposure was similar in both age groups; however, in old mice the growth of the cytokines' levels was more pronounced (especially in the case of IL1). Remodelling of the lung tissue structure did not take place because, despite some progress in the fibrosis score, the hydroxyproline level did not confirm this type of change.

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