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ORIGINAL ARTICLE



Effects of *Aspergillus fumigatus* on glucocorticoid receptor and β 2-adrenergic receptor expression in a rat model of asthma

Xia Zhou^a, Ya-Jie Dang^b, Gui-Fang Wang^a, and Xian-Qiao Jin^a

^aDepartment of Respiratory Medicine, Huashan Hospital Affiliated to Fudan University, Shanghai, China; ^bDepartment of Respiratory Medicine, Shanghai First People's Hospital Affiliated to Shanghai JiaoTong University, Shanghai, China

ABSTRACT

Purpose: Conventional inhaled corticosteroids or β 2-adrenergic receptor agonists do not work well in some asthmatic populations while empirical antifungal therapy has obvious impact on those patients. The study was designed to investigate whether short-term exposure to *Aspergillus fumigatus* (*A. fumigatus*) could decrease glucocorticoid receptor (GCR) and β 2-adrenergic receptor (ADRB2) expression in lung tissue of asthmatic rats. **Materials and Methods:** A rat model of chronic asthma was first established by ovalbumin sensitization and challenge. Rats with chronic asthma were then exposed to short-term application of *A. fumigatus* spores. Airway hyper-responsiveness, eosinophil ratio in bronchoalveolar lavage (BAL) fluid and total IgE in serum were counted in these experimental animals. GCR and ADRB2 expression in the lung were detected and analyzed. Furthermore, the levels of toll-like receptors (TLRs) 2, 3 and 4 in lung tissue were measured. **Results:** Short-term exposure to *A. fumigatus* could down-regulate the expression of GCR, aggravate airway hyper-responsiveness and increase the level of TLR2 in rats with asthma. There were no obvious changes in the levels of ADRB2 expression, recruited eosinophils, total IgE, TLR3 and TLR4 after application of *A. fumigatus* in asthmatic rats. **Conclusions:** These findings indicate that *A. fumigatus* exposure may be involved in glucocorticoids unresponsiveness by down-regulating the expression of GCR in asthmatics. The possibility of *A. fumigatus* colonization or infection should not be ignored in patients of steroid-resistant asthma.

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Aspergillus fumigatus; asthma; glucocorticoid receptor; β 2-adrenergic receptor; toll-like receptors

Introduction

Inhaled glucocorticoids are used widely in respiratory diseases for their anti-inflammatory effects and are regarded as the gold standard of asthma treatment.^[1] With the bronchodilating effect in the airway, inhaled short-acting β 2-adrenergic receptor agonists (β 2-agonists) have been the support of treatment of acute asthma.^[2] However, conventional inhaled corticosteroids or β 2-agonists do not work well in some asthmatic patients. In recent years, a close correlation between increased *Aspergillus fumigatus* (*A. fumigatus*) exposure and loss of asthma control has been noticed.^[3–5] Some randomized controlled trials and case reports had shown that most of patients with refractory asthma or severe persistent asthma were sensitized to fungi and antifungal therapies such as itraconazole improved respiratory symptoms.^[6,7] These findings suggest that *A. fumigatus* participate in the process of therapy-resistant asthma, which

contributes to asthma exacerbation. Therefore, we aimed to explore the possible mechanism by which *A. fumigatus* could decrease drug (glucocorticoids and β 2-agonists) responsiveness.

Glucocorticoid receptor (GCR or Nr3c1) is the main receptor to which glucocorticoids bind. It is a member of the nuclear receptor superfamily to which cortisol and other glucocorticoids bind.^[8] When the ligand is not occupied, GCR acts as part of a protein complex and resides in the cytoplasm. Upon ligand binding, GCR is dissociated from the complex and rapidly translocates into the cell nucleus, where activates GCR dimerizes and binds to so-called glucocorticoid response elements to initiate anti-inflammatory transcription or inhibit pro-inflammatory transcription.^[9,10] Gene network modeling revealed decreased GCR signaling in children with severe therapy-resistant asthma.^[11] Previous studies have reported that reduced number of GCR and altered affinity of

glucocorticoid binding to GCR might contribute to the reduction in glucocorticoid responsiveness.^[12,13]

β 2-adrenergic receptor (ADRB2 or β 2AR) is the main receptor to which β 2-agonists bind. β 2-agonists response is affected by several clinical factors and even genetic polymorphisms in ADRB2 gene.^[2,14,15] ADRB2 is a G-protein coupled receptor expressed in airway smooth muscle and induces bronchial relaxation.^[16] Little attention has been paid to the modulation of ADRB2 caused by airway colonization or infection with microbes. Moore et al.^[17] showed that respiratory syncytial virus infection reduced ADRB2 density and weakened β 2-adrenergic responses in human airway smooth muscle, demonstrating the pivotal role of ADRB2 to β 2-agonists responsiveness.

With the properties described above, GCR and ADRB2 play important roles in the responsiveness of glucocorticoids and β 2-agonists. We further hypothesized that *A. fumigatus* may affect the expression of GCR and ADRB2 in lung tissue of asthmatics which may decrease drug (glucocorticoids and β 2-agonists) responsiveness and lead to therapy-resistant asthma.

Airway inflammation in asthma involves innate immune response and toll-like receptors (TLRs) are thought to be involved in airway inflammation. Research in the recent years has reported the increased expression of TLR2, 3 and 4 and thymic stromal lymphopoietin in fatal asthma, which may contribute to the acute inflammation surrounding asthma deaths.^[18] On the other hand, TLRs are the main group of the pattern recognition receptors (PRRs) involved in recognition of fungi, and TLRs-dependent activation of host cells were shown to play specialized antifungal effector functions in some experimental models of *A. fumigatus* infection.^[19–21] Among them, TLR2 and TLR4 signals have been proved to be important for fungal clearance and both pathways were variably affected by signaling through TLRs, such as TLR3.^[19,22,23] On the basis of these findings, we hypothesized that TLR2, 3 and 4 could be altered in the airways of asthmatics as a result of *A. fumigatus* inhalation.

Materials and methods

Animal experiments

Specific pathogen free, male Wistar rats weighing between 180 and 200 g were supplied by SLRC Experimental Animal Company (Shanghai, China). They

were housed four per cage with food and water available with a 12/12 hour light/dark cycles at constant temperature (23 °C) and relative humidity (40%). All experiments were reviewed and approved by the Medicine Ethical Committee for Laboratory Animals, Fudan University.

A total of 32 male Wistar rats were randomly divided into four groups (n = 8/group): 1) control group (CON); 2) *A. fumigatus*-exposed group (CON + AF); 3) ovalbumin (OVA) sensitized and OVA-challenged group (OVA); 4) OVA-sensitized, OVA-challenged and *A. fumigatus*-exposed group (OVA + AF). Animals were sensitized and challenged as previously described.^[24,25] Briefly, rats were sensitized by an intraperitoneal injection of 1 mg OVA (Albumin Chicken egg, Grade V; Sigma, St. Louis, MO, USA) plus 100 mg aluminum hydroxide (Sigma, USA) in 1 mL normal saline (Baxter Healthcare Company, Shanghai, China) on days 1 and 8. Rats in the CON and CON + AF groups were injected with 1 mL normal saline instead. Then from days 15 to 56, the rats were placed unrestrained in a transparent plastic chamber (20 cm × 40 cm × 50 cm) connected to a nebulizer (AG CLASSIC, 0.2ml/min, MMAD (50%) <5 μ m; Philips, China), and were subjected to bronchial allergen challenge by inhalation of 1% OVA for 30 minutes, 5 consecutive days per week for 6 weeks. Rats in CON and CON + AF groups were challenged by saline instead of OVA. Twenty-four hours after the last challenge, rats in CON + AF and OVA + AF groups were given *A. fumigatus* spores suspension intranasally at 1.0×10^7 spores/mL for 3 days, 200 μ L of suspension per animal (100 μ L to each naris) after anesthesia. Rats were kept erect for 30 seconds, ensuring that the spores entered the trachea and were distributed evenly in both lungs (Figure 1).

Preparation of *A. fumigatus* spores

The strain of *A. fumigatus* (Af293) used in the study was isolated from a patient with invasive pulmonary aspergillosis (IPA). The spores suspension was prepared as previously described in our research.^[25]

Measurement of airway hyper-responsiveness (AHR)

AHR was assessed 24 hours after the last *A. fumigatus* application with a barometric plethysmography (Buxco Electronics Inc., Troy, NY) in

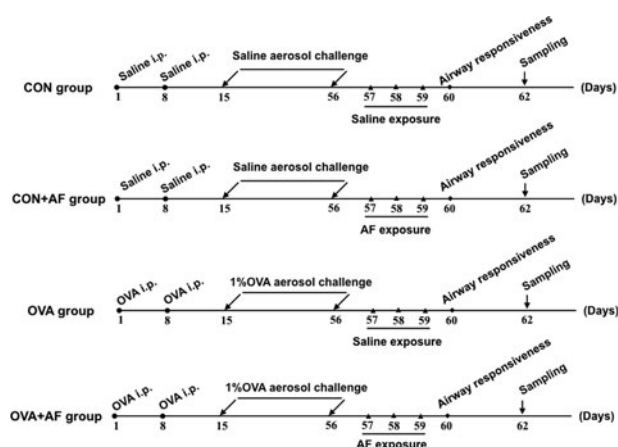


Figure 1. Time schedule of the experimental procedures. Days of sensitization, OVA/ normal saline challenge, *A. fumigatus* exposure and airway responsiveness measurement were indicated. Rats were divided into four groups: (1) CON group: Rats were sensitized and challenged with normal saline; (2) CON + AF group: Rats were sensitized and challenged with normal saline, subsequently, the rats were given *A. fumigatus* spores inhalation; (3) OVA group: Rats were sensitized and challenged with OVA, subsequently, the rats were given normal saline inhalation; (4) OVA + AF group: Rats were sensitized and challenged with OVA, and later be exposed to *A. fumigatus* spores.

response to inhaled methacholine (Sigma-Aldrich, St Louis, MO) as previously described.^[26] Rats were challenged with aerosolized PBS for baseline measurements or with methacholine (3.125, 6.25, 12.5, 25, and 50 mg/mL) to induce bronchoconstriction. Data were recorded and calculated using the dimensionless parameter, enhanced pause (Penh) values.

Bronchoalveolar lavage (BAL) fluid collection and determination of eosinophils percentage

Three days after the last inhalation of *A. fumigatus* spores, rats anesthetized with 2% pentobarbitalum natrium (Merck, Germany) were firstly punctured for blood through their abdominal aorta. The serum was isolated and stored at -80°C until assay. A catheter was inserted into the trachea, and BAL fluid was obtained from the left lung three times with 10 mL PBS totally. The BAL fluid was centrifuged at 1000 rpm for 10 minutes at 4°C ; the supernatant was kept at -80°C until needed; the cells precipitation were resuspended in 200 μL PBS. Percentage of eosinophils was counted by hematology analyzer (SA-3B100123; Mindray Medical, ShenZhen, China).

Detection of total IgE in serum by ELISA

Total IgE in serum was measured with an enzyme-linked immunosorbent assay (ELISA), according to the

manufacturer's instructions (ab157736; abcam, UK). All assays were performed in duplicate, and the mean values were used for statistical analysis.

Western blot analysis of GCR and ADRB2 protein

The lung tissue samples collected were crushed in the frozen state in RIPA lysis buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and 1 mM EDTA) supplemented with protease inhibitors in a tube by shaking with a sterile steel ball (Tissuelyser-24; Shanghai Jingxin Industrial Development co., Ltd, China). Homogenates were centrifuged at 12000 rpm for 10 minutes at 4°C . After that, protein concentrations in the extracted supernatants were detected with BCA protein assay (P0010; Beyotime, Jiangsu, China).

An equal 50 μg protein was loaded per lane onto a 10% SDS-PAGE gel (stacking gel voltage, 90 V for 30 minutes; separating gel voltage, 120 V for 60 minutes). Separated proteins in the gel were transferred to a PVDF membrane (millipore, USA) using a blot transfer system (Bio-Rad Laboratories, USA), 250 mA for 60 minutes. Membranes were blocked for 1 hour in blocking buffer (P0023B; Beyotime, Jiangsu, China), and incubated overnight at 4°C with monoclonal antibody against GCR (1:1000, ab109022; Abcam, UK), ADRB2 (1:1000, ab182136; Abcam, UK) and GAPDH (1:1000, AG019; Beyotime, Jiangsu, China). After washing three times with TBS concentrate (500 mmol/L Tris.HCl; 2.8 mol/L NaCl; 60 mmol/L KCl, Sangon Biotech), the membrane was incubated with a goat anti-rabbit IgG antibody conjugated with horseradish peroxidase (HRP) (1:1000, A0208; Beyotime, Jiangsu, China) at room temperature for 1 hour. The binds were detected by an ECL detection system (P0018; Beyotime, Jiangsu, China). Integrated density values (IDV) were analyzed using a computerized image analysis system (AlphaVIEW SA, FluorChem FC2, Cell Biosciences Inc, USA).

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis of GCR, ADRB2 and TLR 2, 3, 4 mRNA

Total RNA was extracted from the lung tissue samples using the TRIzol reagent (Invitrogen, Life Technologies Corporation, USA) and then was reverse-transcribed using Rever Tra Ace qPCR RT Master Mix (TOYOBO

CO., LTD. OSAKA Japan) as detailed in the manufacturer's guidelines. The following primers were used in the experiments: β -actin, sense 5'-GCA GGA GTA CGA TGA GTC CG-3' and antisense 5'-ACG CAG CTC AGT AAC AGT CC-3'; GCR, sense 5'-GGT GAT TGA ACC CGA GGT GT-3' and antisense 5'-TCA CTT GAC GCC CAC CTA AC-3'; ADRB2, sense 5'-AAG AAT AAG GCC CGA GTG GT-3' and antisense 5'-CAT AAC AGT CGA TGG CTT GC-3'; TLR2, sense 5'-TGT TCC GGG CAA ATG GAT CA-3' and antisense 5'-GCC TGA AGT GGG AGA AGT CC-3'. TLR3, sense 5'-CCC CCT TCC AAC TCC TAT TC-3' and antisense 5'-TCG ATG CAC TGA AAC ATT CC-3'. TLR4, sense 5'-TGG CAT CAT CTT CAT TGT CC-3' and antisense 5'-CAG AGC ATT GTC CTC CCA CT-3'. The 10- μ L PCR reactions consisted of 0.4 μ L of each primer, 5 μ L SYBR Green Mix and 1 μ L Plus, 2.2 μ L ddH₂O, and 1 μ L cDNA. The qRT-PCR program was set as follows: 95°C for 15 s (initial denaturation); 40 PCR cycles: 95°C for 15 s, 60°C for 60 s and 72°C for 45 s (denaturation, annealing and extension); 90°C for 15 s and 60°C for 60 s (fully extension and stableness of the final product). The program was performed using the 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The resulting product of each sample was normalized to that of β -actin transcripts. The quantitative analysis was carried out by the $2^{-\Delta\Delta CT}$ method, and finally processed into fold change of expression.^[27]

Detection of *A. fumigatus* in lung tissue, BAL fluid and whole blood

One hundred mg lung tissues collected were crushed in 1 mL PBS in a tube by shaking with a sterile steel ball. Then, 100 μ L homogenates, BAL fluid and whole blood were grown on potato dextrose agar medium at 37°C, respectively. The positive rate of *A. fumigatus* was detected after 24 hours of culture. If *A. fumigatus* colony was not formed after 7 days of culture, it was considered to be negative.

Periodic acid-silver methenamine (PASM) staining of lung tissue

The right upper lobe was fixed with 10% neutral buffered formalin and paraffin-embedded for histological analysis. Paraffin-embedded tissue was sectioned

at a thickness of 4 mm and stained according to the method described by Rambourg.^[28] The sodium borate, silver nitrate and silver methenamine solutions were freshly prepared. The tissue samples were stained with silver methenamine (at 60°C for 30 minutes) to confirm the presence of *A. fumigatus* spores.

Statistical analysis

All results were presented as means $\pm$ standard deviation (SD). Statistical analyses were performed with SPSS version 20 software (SPSS Institute, Inc., USA). If normality was achieved, one-way analysis of variance (ANOVA) or unpaired t-test was used for the group comparison. In cases where the data was not normally distributed, Kruskal-Wallis ANOVA or Mann-Whitney U test was used. $P < 0.05$ was considered as statistically significant.

Results

Exposure to *A. fumigatus* induces a trend towards increased AHR

As shown in Figure 2, OVA challenge induced typical AHR at each concentration of methacholine, seen from OVA group compared with CON group. There

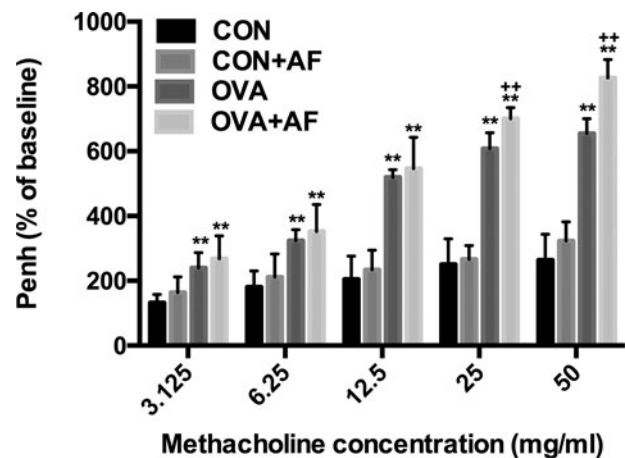


Figure 2. *A. fumigatus* application increased airway hyper-responsiveness in asthmatic rats. Enhanced pause (Penh) values for increasing concentrations of inhaled methacholine were measured in the four groups with whole body plethysmography. Rats were challenged with normal saline for baseline measurements or with methacholine for 2 minutes, and readings were taken and average for 3 minutes after nebulization. All values are displayed as means $\pm$ SD. ** $p < 0.01$ versus CON group; ++ $p < 0.01$ versus OVA group.

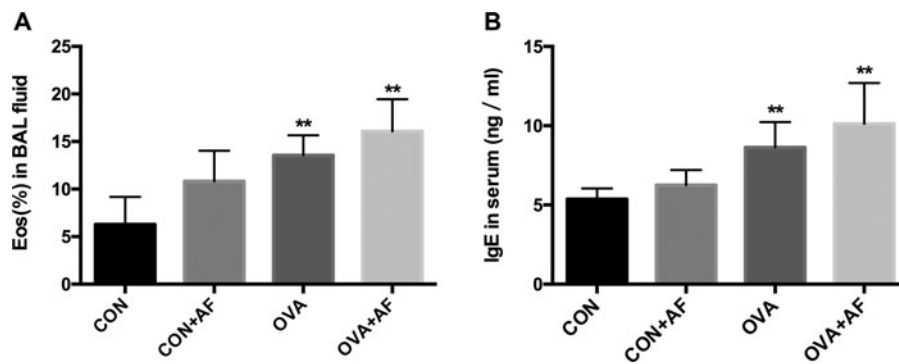


Figure 3. Inhalation of *A. fumigatus* slightly but not significantly increased eosinophils ratio into the airways (A) and total IgE in serum (B) in asthmatic rats. All values are displayed as means $\pm$ SD. ** $p < 0.01$ versus CON group.

was obviously significant difference between OVA + AF group and CON group at each concentration of methacholine. Analysis of the OVA + AF group and OVA group showed that *A. fumigatus* application significantly increased AHR at the concentration of 25 mg/mL and 50 mg/mL methacholine. However, no significant difference in Penh values was observed in CON + AF group when compared with CON group indicating that *A. fumigatus* exposure does not have an effect on airway responsiveness in normal immuno-competent hosts.

As shown in Figure 3, the proportion of eosinophils and total IgE levels in OVA group and OVA + AF group were significantly upregulated when compared with those of CON group. A slight, non-significant increase in the fraction of eosinophils and total IgE levels were showed in OVA + AF group when compared with OVA group. However, *A. fumigatus* application did not influence the proportion of neutrophils, lymphocytes, monocytes and basophils (data not shown).

Effects of *A. fumigatus* exposure on GCR and ADRB2 mRNA and protein expression in lung tissue

The expression of GCR and ADRB2 was examined by qRT-PCR (Figure 4). At the mRNA levels, the expression of GCR mRNA was decreased significantly after *A. fumigatus* application, seen from CON + AF group compared to CON group and OVA + AF group compared to OVA group. There was obviously significant difference between OVA + AF group and CON group on GCR mRNA expression. However, there was no significant difference in the expression of ADRB2 mRNA among those groups.

The presence of GCR and ADRB2 was further confirmed by Western blot analysis (Figure 5). As shown in Figure 5A, the relative expression of GCR protein were decreased in CON + AF group and OVA group when compared with CON group. Marked lower expression of GCR protein was observed in OVA + AF group than those in CON group and OVA group. As depicted in Figure 5B, the levels of ADRB2 protein were similar

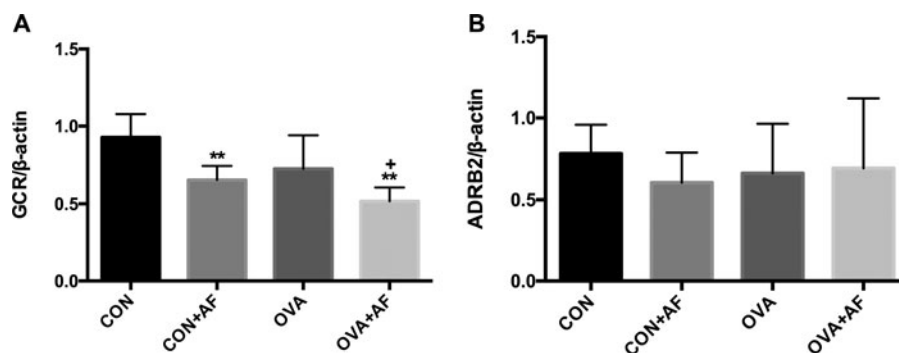


Figure 4. Effects of *A. fumigatus* on GCR and ADRB2 mRNA expression in the lung. *A. fumigatus* down-regulated GCR mRNA expression in the lung in *A. fumigatus*-exposed asthmatic rats (A). The levels of ADRB2 mRNA were similar among the four groups (B). qRT-PCR was performed for mRNA measurement; the resulting product of each sample was normalized to that of β -actin transcripts. The quantitative analysis was carried out by the $2^{-\Delta\Delta CT}$ method, and finally processed into fold change of expression. Data shown were combined results obtained from three to five independent experiments. Each sample was performed in triplicate. All values are displayed as means $\pm$ SD. ** $p < 0.01$ versus CON group; + $p < 0.05$ versus OVA group.

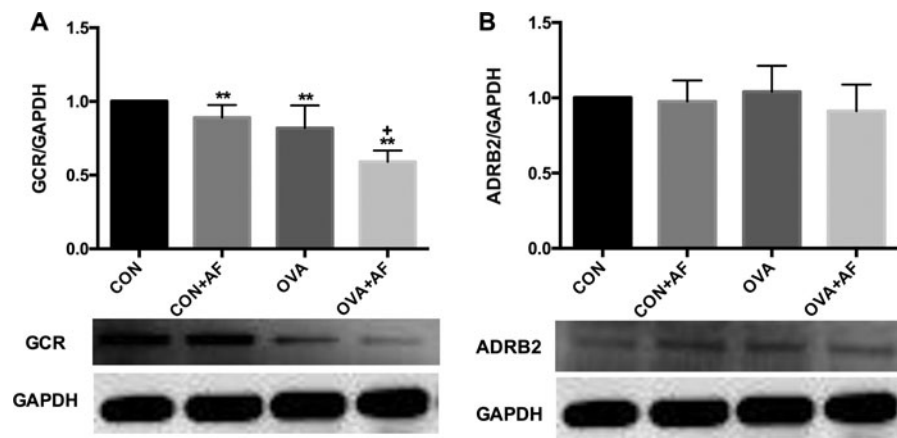


Figure 5. Effects of *A. fumigatus* on GCR and ADRB2 protein expression in the lung. *A. fumigatus* down-regulated GCR protein expression in the lung in *A. fumigatus*-exposed asthmatic rats (A). The levels of ADRB2 protein were similar among the four groups (B). Western blot was performed for protein measurement; GAPDH was used as control; Integrated density values (IDV) were analyzed using a computerized image analysis system. Data shown were combined results obtained from six independent experiments. All values are displayed as means $\pm$ SD. ** $p < 0.01$ versus CON group; + $p < 0.05$ versus OVA group.

among the four groups. Both qRT-PCR and Western blot analyses revealed lower GCR expression levels in *A. fumigatus*-exposed asthmatic rats, suggesting that *A. fumigatus* exposure to asthmatics may be associated with steroid-resistant asthma by down-regulating the expression of GCR.

Effects of *A. fumigatus* exposure on TLR 2, 3, 4 mRNA expression in lung tissue

TLRs are highly expressed in lung tissue and can be activated by either microbial or endogenously derived ligands.^[29] Several cell-wall components of fungi might act as pathogen-associated molecular patterns (PAMPs) that are recognized by TLRs.^[23] To evaluate whether *A. fumigatus* exposure activates the TLRs in lung tissue in asthmatic rats, the levels of TLR2, 3, 4 mRNA were measured by qRT-PCR. As shown in

Figure 6, the levels of TLR2 mRNA was increased upon the exposure to *A. fumigatus*. But OVA sensitization and challenge did not alter TLR2 mRNA expression in lung tissue, seen from CON group and OVA group. In contrast, neither *A. fumigatus* exposure nor OVA sensitization and challenge altered the levels of TLR3 and TLR4 mRNA.

Detection of *A. fumigatus* in lung tissue, BAL fluid and blood

The positive rate of *A. fumigatus* in lung tissue in CON + AF group and OVA + AF group were 0 and 50.0%, respectively. As shown in Figure 7, lung tissue sample in OVA + AF group was positive by culture, and *A. fumigatus* colonies grew on potato dextrose agar medium. According to PASM staining in Figure 8, *A. fumigatus* spores have reached the lung, and the alveoli exhibited

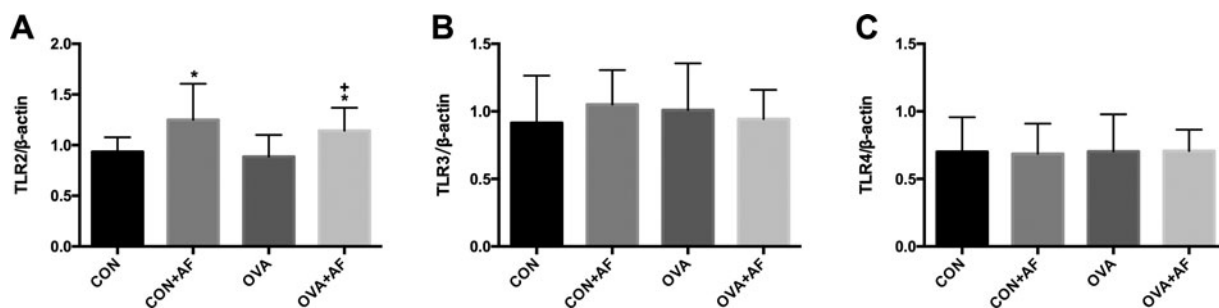


Figure 6. The expression of TLR2, 3, 4 mRNA in the lung in the four groups. qRT-PCR was performed for mRNA expression of TLR2, 3, 4 in the lung. The resulting product of each sample was normalized to that of β -actin transcripts. The quantitative analysis was carried out by the $2^{-\Delta\Delta CT}$ method, and finally processed into fold change of expression. Data shown were combined results obtained from three to five independent experiments. Each sample was performed in triplicate. All values are displayed as means $\pm$ SD. * $p < 0.05$ versus CON group; + $p < 0.05$ versus OVA group.

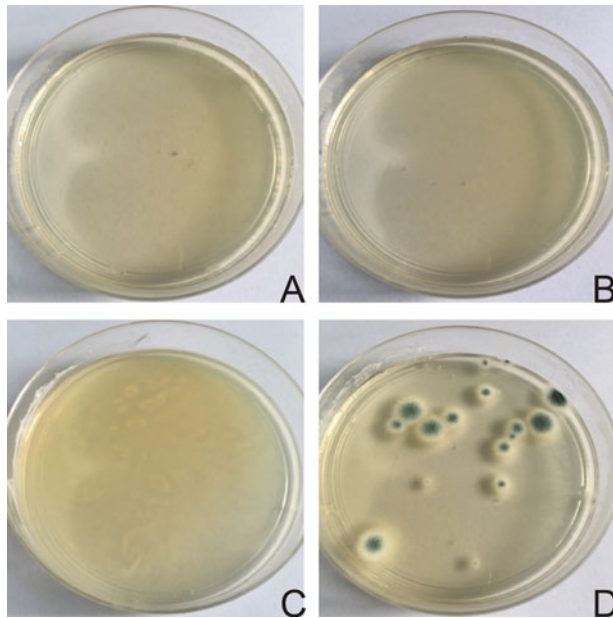


Figure 7. Detection of *A. fumigatus* in lung tissue. Colonies of *A. fumigatus* were formed in OVA + AF group, and other groups were negative by culture. Colonies of *A. fumigatus* were detected by culture on potato dextrose agar medium at 37°C. The positive rate of *A. fumigatus* was detected after 24 hours of culture. If *A. fumigatus* colony was not formed after 7 days of culture, it was considered to be negative. A: CON group; B: CON + AF group; C: OVA group; D: OVA + AF group.

accumulated spores in OVA + AF group. *A. fumigatus* spores were not found in lung tissue in CON + AF group. All lung tissue samples from the unexposed groups were negative by culture. The cultivated result in BAL fluid was the same as that in lung tissue (data not shown). However, colony forming unit was not found in whole blood in the four groups (data not shown). These results suggest that normal immunocompetent hosts can eliminate the inhaled *A. fumigatus* spores timely and the state of asthma increases the chance of *A. fumigatus* colonization in the respiratory tract.

Discussion

Airways colonization by fungi, predominantly by *A. fumigatus*, has been demonstrated in the airway of both asthmatic populations and healthy humans. *A. fumigatus* and related species are distributed extensively in the environment and plenty of studies have shown an association of higher concentration of *A. fumigatus* with asthma exacerbation.^[3,5] In this study, *A. fumigatus*-exposed asthmatic rats showed a trend towards an increased AHR at the high concentration of methacholine challenge, which was in line with the symptom of severe asthma. Our previous studies demonstrated that chronic inhalation of *A. fumigatus* (for 1, 3 or 5 weeks) increased the level of total serum IgE and resulted in the recruitment of eosinophils infiltration into the airway of asthmatic rats in a dose-dependent fashion.^[25] However, the obvious increased production of eosinophils and total IgE were not observed after short-term exposure to *A. fumigatus* in this study.

This is the first study to focus on the influence of short-term *A. fumigatus* exposure or initial *A. fumigatus* colonization on non-allergic bronchopulmonary aspergillosis (ABPA) asthmatic animals while ABPA is associated with long-term *A. fumigatus* colonization in the respiratory.^[30] Rats were sacrificed on 3 days after *A. fumigatus* spores application because *A. fumigatus* spores have reached the lung, and the alveoli exhibited accumulated spores and small presence of hyphae on the 3rd day in immunosuppressed rats.^[31] The culture of *A. fumigatus* in our experiment detected *A. fumigatus* in 4 of 8 (50%) positive lung tissue samples in OVA + AF group. The positive rate of *A. fumigatus* in BAL fluid was in line with the lung tissue samples. Unlike our data, however, in some experimental models of IPA, all lung tissue specimens

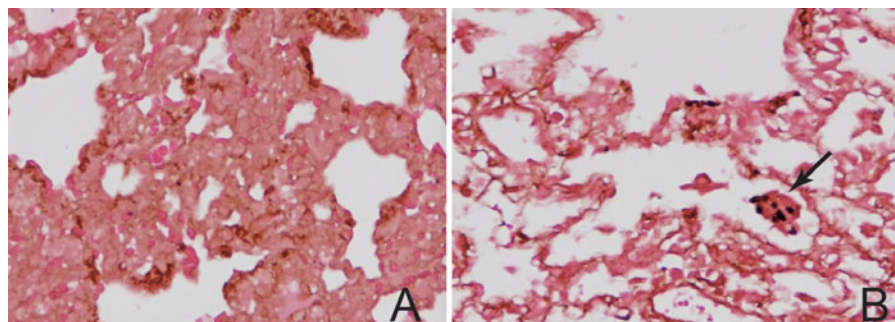


Figure 8. *A. fumigatus* spores in lung tissue (PASM × 400). Periodic acid-silver methenamine (PASM) stained sections from CON + AF group (A) and OVA + AF group (B) showing entire or degraded fungal spores (black material pointed out by arrow) in lung tissue of OVA + AF group.

from immunosuppressed/infection (rats were pre-treated with cortisone or cyclophosphamide before *A. fumigatus* infection) rats were positive for *A. fumigatus* by culture, KOH-calcofluor microscopy and qPCR on the 3rd day post-infection.^[31,32] Moreover, all lung tissue specimens tested in duplicate from CON + AF groups in our study were negative for *A. fumigatus* by culture, and *A. fumigatus* spores have been cleaned up in the lung, seen from PASM staining. Together, it means that normal immunocompetent rats can clear the inhaled *A. fumigatus* spores timely and the state of asthma, as well as immunosuppressed state increases the chance of *A. fumigatus* colonization in the respiratory tract. Apart from lung tissue and BAL fluid, all whole-blood samples from rats in our study were negative for *A. fumigatus* by culture. There are a couple of possible reasons for the negative culture results in this study. First, *A. fumigatus* spores could not migrate into circulatory system due to the relatively short-time exposure (3 days) and host immune defense might limit the migration of *A. fumigatus*. Lin et al.^[31] found that the *A. fumigatus* DNA sequence was not detected in blood samples on day 1 and day 3, but was detected in 50% of the day 5 samples and 66.7% of the day 7 samples in IPA rats, suggesting the spread of *A. fumigatus* infection. Second, the fungal load in blood was low and the method of culture was not sensitive. According to Zhao and colleagues, blood samples from IPA rats failed to demonstrate *A. fumigatus* infection by culture and PCR while samples could elicit positive signals by the real-time nucleic sequence-based amplification method, which was highly sensitive for the detection of *A. fumigatus*.^[32]

The most striking finding of the current study was the down-regulation of GCR mRNA and protein expression in lung tissue in asthmatic rats with *A. fumigatus* exposure. As we know, inhaled glucocorticoids are considered the gold standard of treatments for persistent asthma,^[1] but they are poorly effective in severe asthmatics who present a more corticosteroid insensitive phenotype. Recently, a subphenotype of severe asthma with fungal sensitization (SAFS) has been described in adults.^[33,34] However, mechanisms underlying fungal exposure and severe asthma remain unknown. Defective function of GCR, such as decreased nuclear translocation, protein expression, ligand binding and binding to GRE may be involved in corticosteroid insensitivity in severe asthma.^[35,36] Thus, we hypothesized that fungal-related

steroid-resistant asthma is mediated by the impaired GCR expression. Using asthmatic rats with *A. fumigatus* exposure, we demonstrated decreased GCR mRNA and protein levels in lung tissue in response to *A. fumigatus* exposure compared with those seen in asthmatic rats without *A. fumigatus* exposure. Similar to our study, previous researchers have shown that low GCR expression in peripheral blood mononuclear cells may lead to steroid-resistant nephrotic syndrome in children.^[37] Intracellular GCR expression in T lymphocytes of steroid-resistant children was significant lower than steroids responders.^[9] A significant decrease in GCR expression and binding with dexamethasone has been reported to be involved in tissue resistance to steroids in patients with systemic lupus erythematosus.^[10] These previous studies further certified that impaired GCR expression contributed to steroid resistance in diverse diseases. However, a relationship between fungal exposure and impaired GCR expression has not been reported before. Thus, in our clinical practice in the future, fungal colonization or infection should not be ignored in patients of steroid-resistant asthma.

Similarly, the efficacy of β -agonists is heterogeneous in patients, which is partly due to genetic polymorphism of the ADRB2.^[38] A meta-analysis showed that mutations-induced amino acid changes at positions 16 and 27 altered the regulation of ADRB2, which induced resistance to the smooth muscle relaxation effect of β 2-agonists.^[15] Little attention has been paid to the modulation of ADRB2 caused by airway colonization or infection with microbes. Moore et al.^[17] found that ADRB2 density was reduced in human airway smooth muscle after respiratory syncytial virus infection, which further reduced β 2-adrenergic responses. In contrast, results of the present investigation demonstrated that the expression of ADRB2 mRNA and protein in lung tissue were not influenced by *A. fumigatus* exposure in asthmatic rats.

Airway inflammation in asthma involves innate immune response and TLRs are thought to be involved in airway inflammation. The association between TLRs and allergic diseases has been focus in many human studies and increased expression of TLR2, TLR3 and TLR4 were found in fatal asthma and persistent allergic rhinitis.^[18,39,40] On the other hand, The recognition of fungi is based on the detection of cell wall components by specialized corresponding receptors and TLRs are the main group of the pattern recognition

receptors (PRRs) involved in recognition of fungi.^[21] TLR2- and TLR4-dependent activation of host cells were shown to play a role in cytokine secretion, polymorphonuclear neutrophils activation and susceptibility to *A. fumigatus* infection.^[41–45] Moreover, signaling through TLR2 and TLR4 has been proved to be important for fungal clearance which was variably affected by TLR-activation pathways, such as TLR3.^[19,22,23] Unifies the above studies, we hypothesized that TLR2, 3 and 4 would be altered in the airways of asthmatics after *A. fumigatus* inhalation. Our study demonstrated a significant increase in TLR2 mRNA expression in response to *A. fumigatus* which was in line with the findings described in previous reports. However, the exposure to *A. fumigatus* did not alter the levels of TLR3 and TLR4 mRNAs in our study. According to the previous studies, signaling through TLR2 promoted the fungicidal activity of polymorphonuclear neutrophils through oxidative pathways involving extracellular release of proinflammatory cytokines while TLR4 favored the oxidative pathways through the participation of azurophil, myeloperoxidase-positive granules and IL-10; Both pathways were variably affected by signaling such as TLR3.^[19] Moreover, cytokine secretion induced by *A. fumigatus* might be mediated by TLR2 and TLR4.^[44] Although TLR2 and TLR4 have been demonstrated to be important for fungal clearance,^[19,22,23] the deleterious effect of TLR2 signaling also have been reported, which was associated with an increased survival of CD4 + CD25 + Treg cells, resulting in deficient T-cell immunity and impaired fungal clearance.^[46] In addition, the importance of Dectin-1 is also shown in the defense against *A. fumigatus*^[47,48] and it is likely that Dectin-2 plays an important role in antifungal immunity by Sky-CARD9 innate signaling pathway.^[49] As a whole, innate immune cells express many receptors that can potentially recognize fungi, including TLR2, TLR-4, Dectin-1, Dectin-2, DC-SIGN, SIGN-R1, mannose receptor and Mincle,^[50,51] but in the airway inflammation in asthma, whether the recognition function of these receptors for fungi would be affected is still unknown.

Our study has some limitations. First, we measured the levels of GCR and ADRB2 expression of the lung tissue only at 72 hours, but not at different time points after *A. fumigatus* exposure. Second, we also did not perform quantitative cultures of the lung tissue from *A. fumigatus*-exposed asthmatic rats to ascertain fungal burden and other organs were not studied for

the presence of *A. fumigatus* to determine whether infection from the lungs had spread to any other organs. We just initially explored the possible recognition receptors that potentially can identify *A. fumigatus*. Whether TLR2 has protective or detrimental effects in asthmatic models with *A. fumigatus* exposure or colonization is still unknown.

In conclusion, the present study showed that *A. fumigatus* exposure to asthmatics could down-regulate the expression of GCR mRNA and protein in lung tissue and aggravate AHR; Thus, *A. fumigatus* might be involved in glucocorticoids unresponsiveness by down-regulating the expression of GCR, leading to steroid-resistant asthma. The special emphasis should be placed to the possibility of *A. fumigatus* colonization or infection in the respiratory tract in clinical cases of steroid-resistant asthma.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

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