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Activation of TLRs/NF-κB signaling pathway and production of diverse cytokines in the incidence and development of invasive pulmonary aspergillosis

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Aspergillus fumigatus **is a common saprophytic fungus in the air. It can cause a severe infectious disease called invasive pulmonary aspergillosis (IPA). To establish the activation of TLRs/NF-κB signaling pathway in invasive pulmonary aspergillosis (IPA) model of wild type mouse, three groups of mouse were used in the study; the normal mice; mice infected with *A. fumigatus* conidia; IPA mouse infected with *A. fumigatus* conidia. *A. fumigatus* burden in lung tissue and lung pathology were detected and evaluated in IPA mouse. The levels of TLR2, TLR4 mRNA, and some inflammatory cytokines were investigated. (1) Severe inflammatory responses were found in IPA mice after nose inhalation of *A. fumigatus* conidia 72 h; In addition, *A. fumigatus* burden in IPA group was higher than that of normal mice with infection in all time. (2) Compared with normal mice with infection, IPA group displayed a low level of TLR2 mRNA in the early stage of infection, whereas it was strongly expressed in the late stage (120 h and 144 h), the expression level of TLR4 mRNA was always low during *A. fumigatus* infection; interestingly, the expression level of NF-κB p65 protein was quickly increased in the early stage (24 h) of infection, and then continuously declined. (3) Normal mice with *A. fumigatus* inoculation expressed high levels of pro-inflammatory cytokines (TNF-α, IL-1β) in the early stage of infection. The highest expression levels appeared at 48 or 72 h, and then declined to normal level. Simultaneously, anti-inflammatory cytokine IL-10 protein was elevated in the late stage of infection. However, IPA mice had a lot secretion of anti-inflammatory cytokine IL-10 protein the early stage of infection, and then displayed a significant decrease in the late stage, whereas the pro-inflammatory cytokines (TNF-α, IL-1β) was slowly secreted in low levels. The abnormal activation of TLRs/NF-κB signaling pathway induced the loss of balance between pro-inflammatory and anti-inflammatory cytokines, and eventually leads to the incidence and development of IPA.**

Key words: *Aspergillus fumigatus,* IPA, TLRs/NF-κB, cytokine, pathogenesis.

INTRODUCTION

Aspergillus fumigatus is a common saprophytic fungus in the air. It has a small diameter and can be passively inhaled into the respiratory tract. *A. fumigatus* conidia will not result in disease inhaled by healthy people, for it can be eliminated by innate immune system in the lungs. However, in hosts with impaired immunity, it can cause a

severe infectious disease called invasive pulmonary aspergillosis (IPA). The mortality rate of IPA ranged from 60 to 94% (Jeurissen et al., 2012; Slavin et al., 2015; Bassetti et al., 2018; Botero et al., 2018). Hitherto, the pathogenesis of IPA is not well illuminated. Toll-like receptors (TLRs) is one major pattern-recognition receptors (PRRs) involved in the early host defence against pathogen invasions (Slavin et al., 2015). By recognizing pathogen-associated molecules, TLRs can trigger corresponding signal transduction, activate host immune system, and remove invaded pathogens.

Wang et al. (2001) firstly reported the function of TLRs in response to *A. fumigatus* (Wang et al., 2001). After that, a lot of *in vitro* studies showed TLRs, mainly TLR2 and TLR4, played roles in immune responses in *A. fumigatus* infection. However, contradicted results still existed in current studies. Meier et al. (2003) indicated both TLR2 and TLR4 involved in response to *A. fumigatus* stimulation. Similarly, *in vivo* studies also showed inconsistent even opposite results. By using $TLR2^{-/-}$ and $TLR4^{-/-}$ mice immunosuppressed with cyclophosphamide, Bellocchio et al. (2004) elucidated that it was TLR4 rather than TLR2 involved in host immune response to *A. fumigatus.* On the contrary, Balloy et al. (2005) showed the key role of TLR2 in immune responses stimulated by *A. fumigatus* using $TLR2^{-/-}$ mice with reduced neutrophils caused by vincristine treatment. Although $MyD88^{-/-}$ mice were more sensitive to *A. fumigatus* infection compared with control or single TLR knockout mice, Dubourdeau et al. (2006) argued that TLR2, TLR4 and MyD88 were not necessary for the elimination of *A. fumigatus*. By use of gene knockout mice and *in vitro* transfection, current studies usually focused on the investigation of TLR functions in a single time point during *A. fumigatus* infection (Wu et al., 2015; Rizzetto et al., 2013; Carvalho et al., 2012).

We established IPA model of wild type mouse. With dynamic investigation of the expression levels of TLR2 and TLR4 mRNA, the protein levels of NF-κB p65, and the levels of inflammatory cytokines including TNF-α, IL–1β and IL-10 protein in pulmonary tissues, together with evaluation of the *A. fumigatus* dosage, and the lung pathology, we elucidated the functions of both TLRs/NF-κB signaling and its multiple downstream cytokines in the development of IPA. This study will provide an insight into the pathogenesis of IPA.

MATERIALS AND METHODS

Experimental animals and grouping

BALB/c SPF mice (Certificate of Conformity: SCXK 2003-0002, male, 6 to 8 weeks old, 20~25 g) were provided by Shanghai SINO-BRITISH SIPPR / BKLAB animal center. Mice were divided into three groups randomly, 25 rats in each group: (1) Normal Group (normal mice); (2) Normal mice with infection (N + *A. fumigatus*); (3) IPA Model Group (normal mice were given with Immunosuppressant and inoculated with *A. fumigatus*).

Strain and culture medium

A. fumigatus (clinical isolates, Separate No. 3910): was purchased from the Fungal Culture Collection of Chinese Medicine Centre (Nanjing). Cells were cultured in Czapek's medium at 26°C.

Main reagents

Cyclophosphamide (CY, NO.: 06060521) was purchased from Jiangsu Hengrui Medicine Co., Ltd.; Trizol reagent was from invitrogen company; TaKaRa RNA PCR Kit 3.0 (AMV) Kit was purchased from Dalian TaKaRa Biotechnology Co., Ltd.; antibodies (Rabbit anti-NF-κB p65, Rabbit anti-IL-1β, Goat anti-rabbit HRP secondary antibodies) were purchased from Santa Cruz Biotechnology (Beijing, China). Polymerase chain reaction (PCR) primers were from Shanghai Biological Engineering company; ultrapure water (UPW, NO.: 07020201) was from U.S. MIUIOORE Inc..

IPA model of mice

According to the literature, the method was given as follows: BALB/c mice were injected intraperitoneally with 100 $mg \cdot kg^{-1} \cdot d^{-1}$ of CY within 2 days. Whereafter, mice were administered intranasally with 50 μL (Concentration: 10^7/mL) spore suspension of *A. fumigatus*. In order to maintain the effect of immunosuppression, mice were given additional CY (100 $mg \cdot kg^{-1} \cdot d^{-1}$) when inoculated with *A. fumigatus* at 96 h (Tang et al., 1993).

Collection and processing of specimen

Mice with nose inhalation of *A. fumigatus* conidia were sacrificed at different time points of 24, 48, 72, 120 and 144 h (5 mice at each time point), then lung tissue were isolated in sterile manner, and conserved in -80°C refrigerator.

A. fumigatus colony counting of lung tissue

100 mg of lung tissue were taken and made into 10% homogenate, then 0.1 ml of it was inoculated on Czapek's medium after diluting 100 times, counting colony after 5 days.

Lung tissue pathology

Observing histological injury and spore germination after all the mice produced paraffin sections of lung tissue and conventional HE staining.

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Table 1. Primer sequences and polymerase chain reaction (PCR) reactive conditions.

Gene	Name	Sequence	temperature (°C)	product (bp)
TLR2	Upstream: downstream:	5'-ACCTCCCTTGACATCAGC-3' 5'-TCGTACTTGCACCACTCG-3'	59	902
TLR4	Upstream: downstream:	5'-ATCTGGTGGCTGTGGAGAC-3' 5'-TTCCCTGAAAGGCTTGGTC-3'	59	288
β-tublin	Upstream: downstream:	5'-AAGGGTCACTACACCGAG-3' 5'-GCGAATCCTGGCATGAAGAAGT-3	59	506

Table 2. Mice lungs burdern of *A. fumigatus* ($\bar{x} \pm s$, n = 5, Unit: CFU/mL).

	Time (h)				
Group	**24**	**48**	**72**	**120**	**144**
Normal mice with infection	1605 ± 298.8	1738 ± 254.0	210 ± 43.2	78 ± 11.3	36 ± 5.2
IPA Group	25800 ± 5533*	28100 ± 6129*	15750 ± 3096*	17500 ± 2543*	9500 ± 1011*

*p<0.05, vs normal mice with infection.

Detection of the target genes expression of lung tissue by RT-PCR

The expression of mRNA was determined using reverse transcription polymerase chain reaction (RT-PCR) analysis. After conidia stimulation, cells were homogenized with 1 ml Trizol reagents, and 1 µg of total RNA was reverse transcribed with a RT-PCR kit (TaKaRa Biotechnology Co., Ltd.) in accordance with the manufacturer's instructions. PCR was performed in tubes using PTC-200 DNA Engine Cycler. After extraction of total RNA and construction of cDNA, PCR amplification using EC3 Chemi HR410 Imaging System, and PCR products were determined using Bandscan image analysis software. The expression of the corresponding target gene was standardized against β-actin. Specific primers for PCR analysis were synthesized using ProTaq DNA polymerase (TaKaRa Biotechnology Co., Ltd.) (Table 1). Values were normalized to β-actin gene expression and are expressed relative to the control group. Primers and PCR reaction conditions are listed in Table 1.

Detection of the target proteins expression of lung tissue by Western blot

Firstly, nuclear protein and total protein from 100 mg lung tissue was extracted; secondly, SDS-PAGE electrophoresis was carried out and transferred to semi-dry membrane; and again, incubated with the corresponding primary antibody (1:250) and secondary antibodies (1:8000) at 37°C for 1 h and finally, the film were exposed to X-ray after colouring with ECL.

Statistical methods

The values of optical density scanning of target band which was measured in the agarose gel and X-ray film by image analysis software Bandscan were read. Afterwards, the expression of its corresponding target gene and protein were respectively standardized by scanning values in each group of β-tublin and β-actin bands. Here, each experiment was repeated three times, and results were indicated as $\bar{x} \pm s$. Statistical software SPSS 10.0 was used to conduct t-test analysis and significant difference was determined at P<0.05.

RESULTS

Morphological analysis of lung pathology

Seventy-two hours after inoculation with *A. fumigatus,* compared with normal mice, the alveolus space in normal mice with infection enlarged, accompanied with inflammatory responses including inflammatory cell infiltration and hemorrhage injury (Figure 1A and B); as a comparison, IPA group had a lung abscess and severe hemorrhage (Figure 1C); in addition, airway epithelial desquamation and mycelium formation were also observed in the IPA group (Figure 1C).

Assessment of *A. fumigatus* burden in pulmonary tissue

The CFU assay indicated that pulmonary tissue from the IPA Group had heavy *A. fumigatus* load (p<0.05) (Table 2) compared with normal mice with infection. As a contrast, normal mice without *A. fumigatus* inoculation showed negative signal in this assay.

Investigating the expression levels of TLR2 and TLR4 mRNA in mice pulmonary tissue

The mRNA levels of TLR2 and TLR4 with RT-PCR were

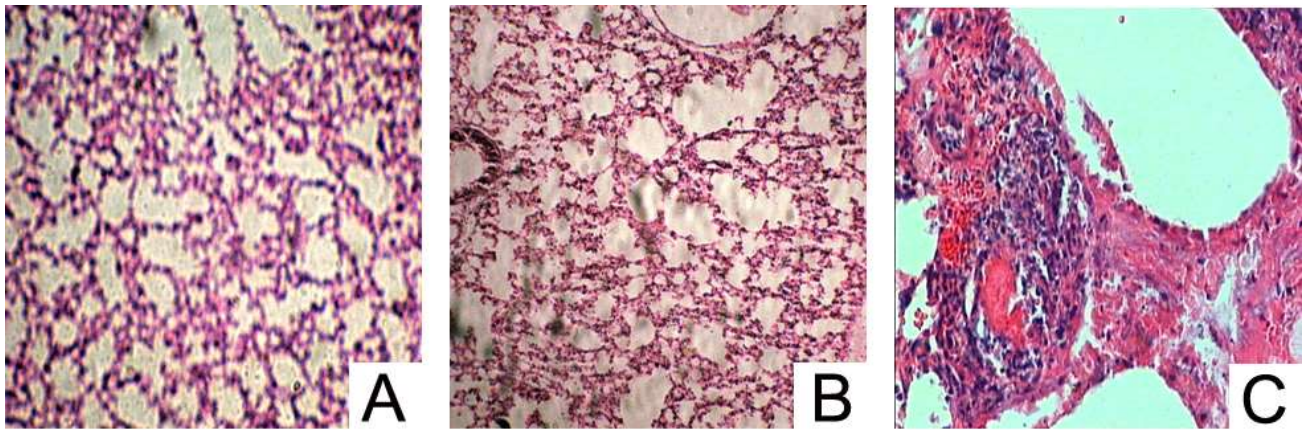

Figure 1. HE staining of the lungs at 72 h after inoculation with *A. fumigatus* (× 200). A, Normal group; B, normal mice with infection; C, IPA Group.

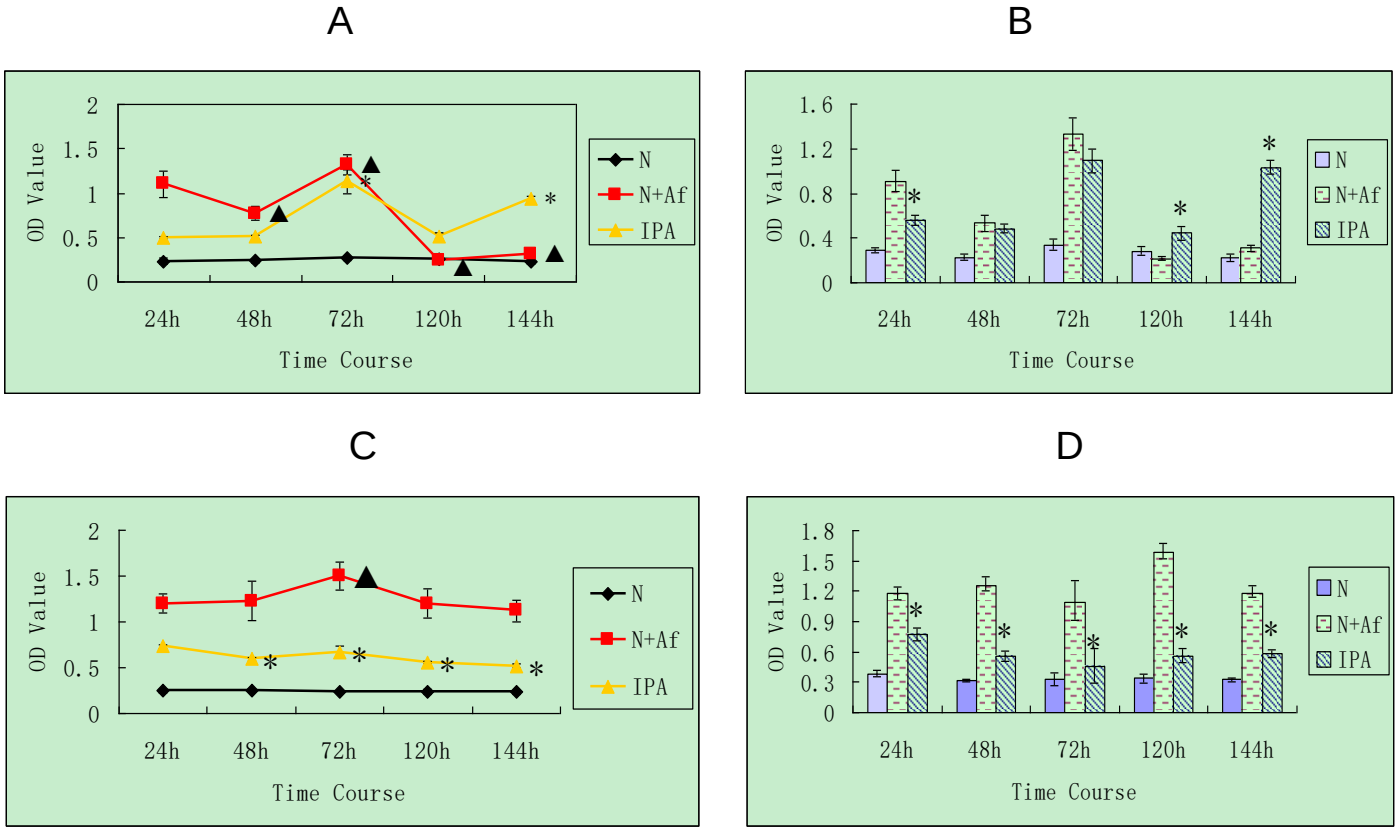

Figure 2. The expression levels of TLR2 and TLR4 mRNA in mouse pulmonary tissue. N, Normal Group; N+ *A. fumigatus;* normal mice with infection; IPA, IPA Group. **A.** The expression of TLR2 mRNA at different time. ▲ $p<0.05$, vs at 24 h in N+ *A. fumigatus* Group; *$p<0.05$, vs at 24 h in IPA Group. **B.** The expression of TLR2 mRNA at the same time point of each group. *$p<0.05$, vs N+ *A. fumigatus*. **C.** The expression of TLR4 mRNA at different time. ▲$p<0.05$, vs at 24 h in N+ *A. fumigatus* Group; * $p<0.05$, vs at 24 h in IPA Group. **D.** The expression of TLR4 mRNA at the same time point of each group. Note:* $p<0.05$, vs N+ *A. fumigatus*.

tested by time course (Figure 2). Compared with normal group, TLR2 mRNA in normal mice with infection was highly expressed before 72 h and then decreased. Until 120 h later, the expression of TLR2 mRNA recovered to a normal level. However, IPA group constantly presented high expression ($p<0.05$); in contrast with normal mice

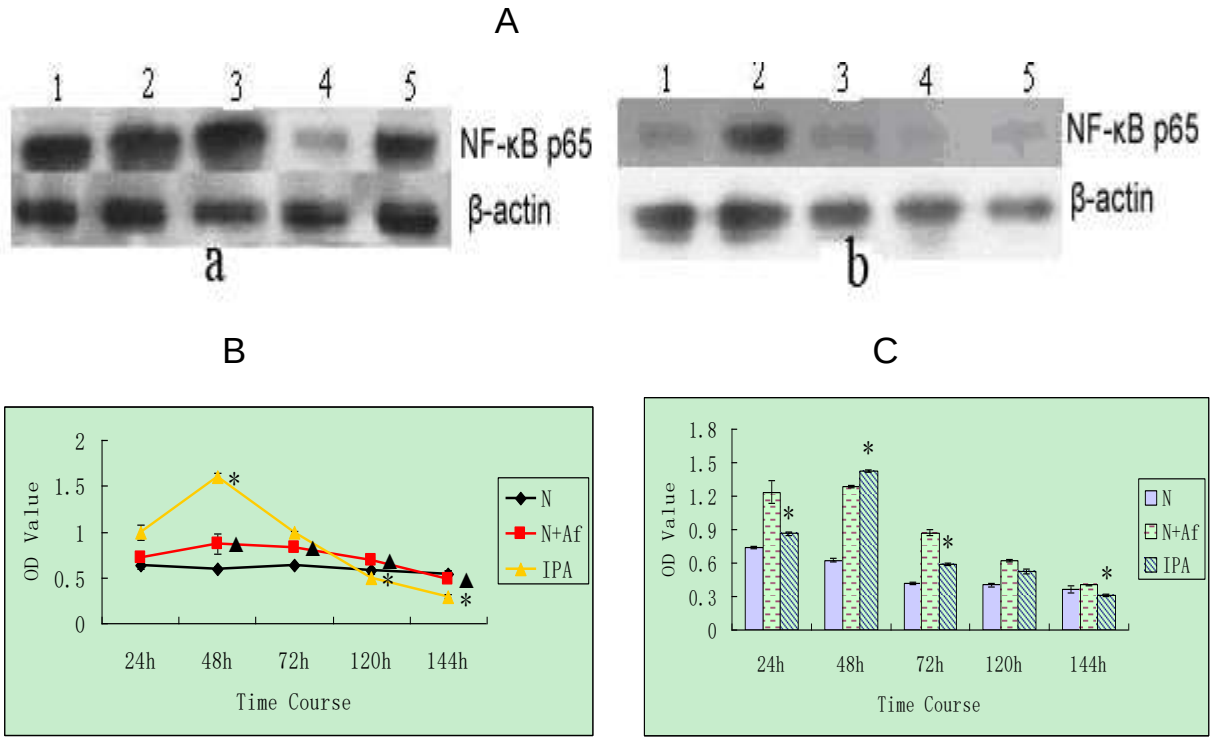

Figure 3. The expression of NF-κB p65 protein. **A.** Western--Blot bands of NF-κB p65 at different time. a. N+ *A. fumigatus*; b. IPA Group. 1: 24 h; 2: 48 h; 3: 72 h; 4: 120 h; 5: 144 h. **B.** The expression of NF-κB p65 at different time. ▲$p<0.05$ vs at 24 h in N+ *A. fumigatus* Group; * $p<0.05$ vs at 24 h in IPA Group. **C.** The expression of NF-κB p65 at the same time point of each group .Note： * $p<0.05$, vs N+ *A. fumigatus*.

with infection, IPA group had a low expression level of TLR2 mRNA in the early stage of infection (24 h), whereas its expression reached a high level in the later stage (120 and 144 h) (Figure 2).

As shown in Figure 2C and D, during the infection of *A. fumigatus,* the expression of TLR4 mRNA in normal group and IPA group showed an increasing status; in comparison with normal mice with infection, the expression of IPA group displayed a continual lower level. By analysis of the altered expression levels of TLR2 and TLR4 mRNA in different mouse group, the results showed that TLR2 mRNA in IPA group was lowly expressed in the early stage of *A. fumigatus* infection (24 h) but strongly expressed in the late stage (120 and 144 h), while the expression levels of TLR4 mRNA remained in a low level during infection.

Analysis of the expression of NF-κB p65 protein

Time course experiments were conducted to evaluate the expression of NF-κB p65 protein in mouse pulmonary tissues from different treatment groups. As shown in Figure 3, NF-κB p65 protein levels were measured by western blot. Twenty-four hours after inoculation with *A. fumigatus,* the expression of NF-κB p65 protein of normal group gradually increased, and then decreased to normal levels after 72 h. On the other hand, IPA mice had a sharp elevation of NF-κB p65 protein after 24 h. It peaked at 48 h, followed by a decline in a level lower than normal mice with infection ($p<0.05$) (Figure 3B and C).

Measurement of TNF-α, IL-1β and IL-10

The expression of inflammatory cytokines was also measured by Western--Blot.

Analysis of the expression of TNF-α protein

TNF-α in infected normal mice displayed a strong expression, which reached the highest level at the time point of 72 h and restored to normal range. As a comparison, IPA mice had a lower TNF-α expression with mild alternation (Figure 4A and B).

Analysis of the expression of IL-1β protein

IL-1β in IPA mice was lowly expressed and began to gradually increase until 48 h later, which displayed a

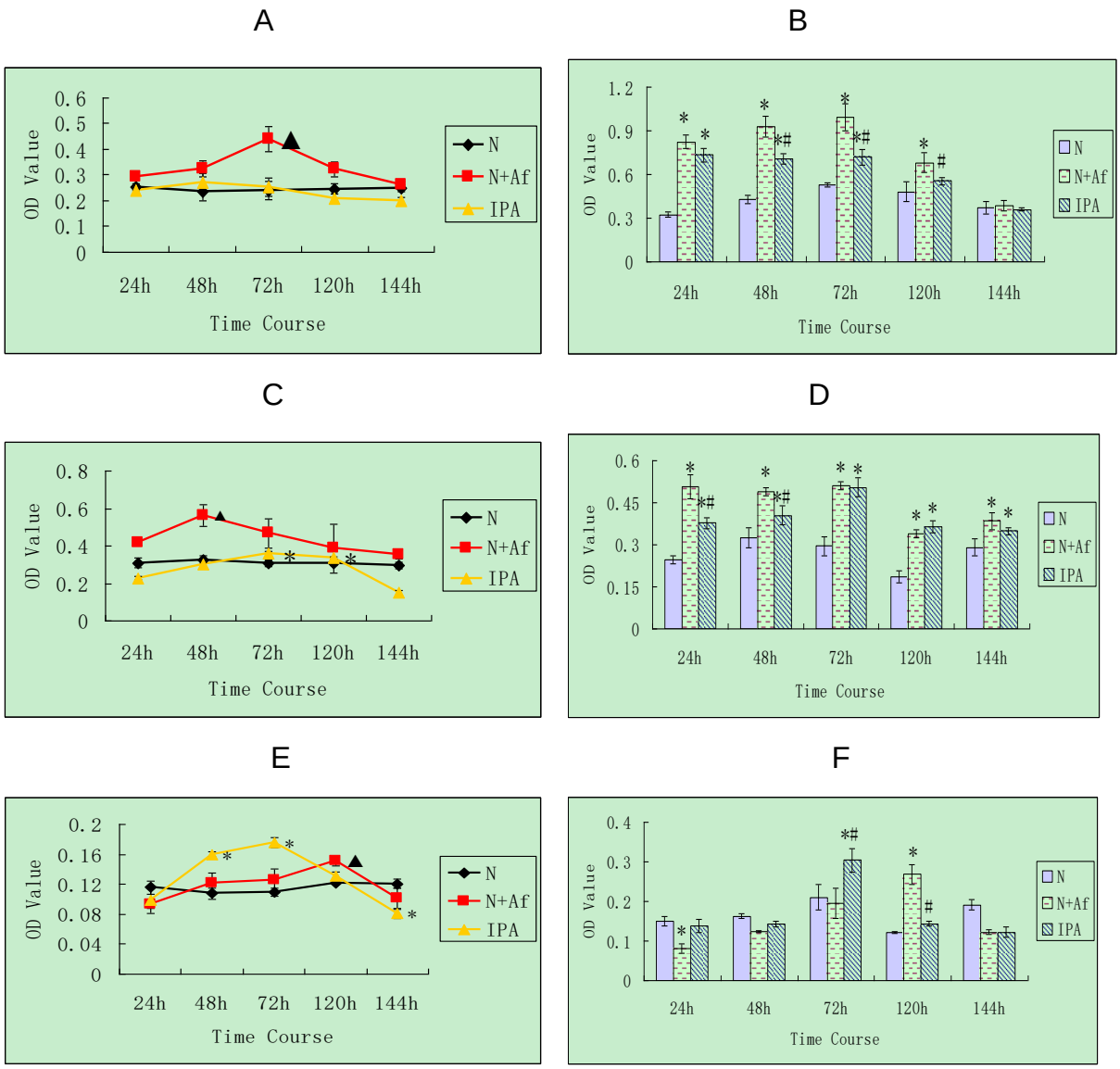

Figure 4. The expression of TNF-α,IL-1β and IL-10. **A.** The expression of TNF-α at different time.▲p<0.05, vs at 24 h in N+ *A. fumigatus* Group; * p<0.05, vs at 24 h in IPA Group. **B.** The expression of TNF-α at the same time point of each group. * p<0.05,vs N; # p<0.05, vs N+ *A. fumigatus*. **C.** The expression of IL-1β at different time. Note:▲p<0.05, vs at 24 h in N+ *A. fumigatus* Group; * p<0.05,vs at 24 h in IPA Group. **D.** The expression of IL-1β at the same time point of each group. Note：* p<0.05, vs N; # p<0.05, vs N+ *A. fumigatus*. **E.** The expression of IL-10 at different time. ▲p<0.05,vs at 24 h in N+ *A. fumigatus* Group; * p<0.05, vs at 24 h in IPA Group. **F.** The expression of IL-10 at the same time point of each group. Note：* p<0.05, vs N; # p<0.05, vs N+ *A. fumigatus*.

decrease expression after 120 h (p< 0.05). On the contrary, the normal mice with *A. fumigatus* infection had a higher expression of IL-1β, which fleetly was elevated and peaked at 48 h (p < 0.05) and then gradually declined (Figure 4C and D).

Analysis of the expression of IL-10 protein

IL-10 in infected normal mice displayed a low expression level and gradually increased and then peaked at the time point of 120 h (p < 0.05) whereas IPA had a strong IL-10 expression in the early stage of infection (before the time point of 72 h) and then reduced to a normal level (Figure 4E and F).

DISCUSSION

Effective innate immunity is the first line of defense to *A.*

fumigatus infection. Phagocytosis of the alveolus macrophages kills inhalational conidia and prevents the formation of hyphae, which can colonize in the host and are associated with lethal infection. Once the conidia escape from phagocytosis and develop into hyphae, neutrophils will take over the defense line. At the same time, macrophages and lung dendritic cells phagocytize conidia and hyphae, present antigens and initiate T cell immune response. Innate immunity not only confers the first line of defense in resistance to *A. fumigatus* infection, but also provides specific signals for initiation of adaptive immunity (Bassetti et al., 2018; Wu et al., 2015). However, the mechanism of innate immunity against the infection of *A. fumigatus* is still largely unknown.

To activate host defense and eliminate invasive pathogens, innate immune response is initiated by pattern recognition, a conserved and pathogen-specific molecular recognition pattern mediated by a series of PRRs that is widely express in macrophages and various cell types. TLRs, the most studied PRRs, belong to type I transmembrane receptors, which broadly is distributed in monocyte-macrophage system, endothelial cell and dendritic cell, etc. By recognizing the associated molecular pattern of invasive pathogens, TLRs activate innate immune response and induce adaptive immune response. TLRs trigger the activation of NF-κB 65 signaling and a series of other downstream networks, leading to the secretion of multiple cytokines and the induction of related biological effects (Portou et al., 2015; De Nardo, 2015; Kang et al., 2018).

Here, in order to systematically mimic patient IPA, the dynamic alternations of TLRs, NF-κB and its downstream cytokines were evaluated in both normal and immunodeficient mice during infection of *A. fumigatus*. Also, cyclophosphamide was used to induce immunosuppression of mice. And, the pathological alternation of pulmonary tissues and culture of *A. fumigatus* was compared in normal mice with those in immunosuppressive mice after nose inhalation of *A. fumigatus* conidia.

Results showed that immunosuppressed mice with nose inhalation of *A. fumigatus* presented pathological alternations similar to clinical IPA cases, indicating the successful establishment of mouse IPA model. Analysis from pulmonary histology combined with CFU assay reminded us that immunosuppressive mice were not able to effectively initiate immune responses, which caused late inflammatory reactions in the early stage for elimination of conidia and suppression of hyphae growth. On the contrary, overreacted inflammatory responses in the late stage of infection led to severe damage of lung tissues.

TLRs/NF-κB signaling is the important network for the regulation of inflammatory and immune response, and also the major pathway for resistance to infection. In this study, we discovered the different dynamic expression pattern of TLR2 and TLR4 mRNA between IPA Group and normal mice with infection. TLR2 mRNA in IPA Group was lowly expressed in the early stage of *A. fumigatus* infection (24 h) but strongly expressed in the late stage (120 and 144 h), while the expression levels of TLR4 mRNA stayed in a low level during infection. Normal mice with infection had a high expression of TLR2 mRNA before 72 h and decreased after that. Until 120 h later, the expression of TLR2 mRNA recovered to a normal level while TLR4 mRNA had a constitutive strong expression during the infection. These results indicate that TLR2 mRNA was abnormally activated in the late stage of IPA, while TLR4 mRNA was always suppressed. We also found that the expression of NF-κB p65 proteins were opposite in these two groups. Twenty-four hours after *A. fumigatus* inoculation, normal mice displayed a gradually increased NF-κB p65 protein, and it declined to a normal level after 72 h; whereas IPA mice had a sharpened increase of NF-κB p65 protein in 24 h, which peaked at 48 h and quickly decreased to a level lower than the average. Previous study showed that the transduction of TLR signaling was under fine regulation of some factors in the normal host, and the produce of NF-κB negatively regulated the activation of TLRs and ensured appropriate intensity of TLR signaling by this complicate feedback mechanism (Carmody et al., 2007). Our results imply a disorder of the regulatory feedback network of TLRs/NF-κB in the IPA host.

Cytokines, an important kind of secretive immune molecules, play roles in diverse biological functions including regulation of cell physiology, mediation of inflammatory responses, involvement of immune reactions, and repair of tissues, etc. The different functions of various cytokines are closely related with the situation and progression of infectious diseases (Peck and Mellins, 2010). During the infection of *A. fumigatus*, there exists a subtle balance between different cytokine types. For an instance, TNF-α, IL-12 and IFN-γ confer resistance to *A. fumigatus* infection in mouse experiment, whereas IL-4 and IL-10 function in opposite direction. In addition, IPA patients went worse when using antibodies of TNF-α and IFN-γ (Rivera et al., 2006). Serum with a high ratio of IFN-γ/IL-10 from clinical IPA patient showed better anti-fungal effects (Hebart et al., 2002). Serum ELIPOT analysis showed *A. fumigatus* -specific T cell in the IPA patient with leukemia and neutropenia produced high levels of IL-10 and low levels of IFN-γ (Potenza et al., 2008). Chronic granulomatous disease (CGD) patient had high levels of pro-inflammatory cytokines after *A. fumigatus* infection compared with normal host, indicating that the disorders of inflammatory and anti-inflammatory responses resulted in increased IPA susceptibility (Shalit et al., 2006).

The disordered activation of TLRs/NF-κB will inevitably lead to abnormal secretion of its downstream cytokines. In this study, we investigated the dynamic alternations of pro-inflammatory cytokines (TNF-α and IL-1β) and anti-inflammatory cytokine (IL-10).

Results show that the expression levels of cytokines (TNF-α, IL-1β and IL-10) in mouse lung were closely correlated with pulmonary pathological impairment. Normal mice with *A. fumigatus* inoculation displayed high levels of pro-inflammatory cytokines (TNF-α and IL-1β) in the early stage of infection. Their expression levels all peaked at 48 or 72 h, and thereafter declined to normal level. At the same time, anti-inflammatory cytokine IL-10 elevated in the late stage of infection. Moreover, lung pathology results showed obvious hyperemia and hemorrhage appeared before 72 h, and thereafter inflammatory responses were gradually alleviated, which indicated that secretion of pro-inflammatory cytokines (killing inhalational conidia and preventing the formation of hyphae) was the major inflammatory responses in the early stage of *A. fumigatus* infection, whereas secretion of anti-inflammatory cytokine in the late stage protected tissues from severe impairment caused by overreacted inflammatory responses. In contrast, a lot of secretion of anti-inflammatory cytokine IL-10 in the early infectious stage of IPA mice and a significant decrease of it in the late stage, combined with slow and low secretion of pro-inflammatory cytokines (TNF-α and IL-1β), caused obstacle in timely elimination of conidia and prevention of hyphae formation. Furthermore, excessive secretion of pro-inflammatory cytokines induced overreaction of inflammatory responses and consequent severe lung injury.

Inflammation is one of the necessary parts of effective immune responses in resistance to IPA. Appropriate inflammatory responses can availably eliminate local *A. fumigatus*, whereas improper or overreacted inflammatory responses will cause IPA and associated lung injury (Romani and Puccetti, 2007). Effective inflammatory responses depend on the mutual cooperation or restriction between diverse immunocytes, which ultimately help the host eliminate exotic antigens as well as protect its own tissues by the regulation of the secretions and functions of diverse cytokines. Recognition of pathogens by PRRs is the key of innate and adaptive immunities. Multiple regulations ensure complicate but appropriate activation of signaling pathways. Abnormal activation of upstream and midstream molecules in signaling pathways will affect their downstream networks, and finally cause inflammatory diseases (Kang et al., 2018).

The results indicates that the disorder of TLRs/NF-κB signaling pathway in the immunosuppressed mice with *A. fumigatus* inoculation causes the loss of balance between pro-inflammatory and anti-inflammatory cytokines and eventually led to the incidence and development of invasive aspergillosis.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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