Full Length Research Paper

Changes of nucleotide-binding oligomerization domains (NODs) signaling pathway in the incidence and development of invasive pulmonary aspergillosis

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This study investigates the effect of nucleotide-binding oligomerization domains (NODs) signal pathway in invasive pulmonary aspergillosis. Mice were randomly divided into three groups: 1) normal mice (control group), 2) normal mice infected with Aspergillus fumigatus, 3) normal mice treated with immunosuppressant and inoculated with A. fumigatus (IPA Model). Mice were sacrificed at different time points after inhaling A. fumigatus spores by nose. Their lungs were extracted under sterile condition, and were used to count the fungal colonies; and also the pathological sections of lungs were observed by HE staining. RT-PCR was used to detect the expression of the NOD1, NOD2 and RIP2 mRNA of mice lung. Western blot was used to detect the expression of TNF-α. 72 h after inhaling A. fumigatus spores, a large number of hyphae and severe inflammation were found in the lung of IPA model mice group; and the lung burden of IPA mice were more than that of normal+IPA. fumigatus group at each time points. When compared with normal+IPA. fumigatus group, the expressions of NOD1 and RIP2 mRNA were persistently descending in IPA model mice group; the expression of NOD2 mRNA was abnormally raised in early stage of infection (24 h), then decreased in the later stage. However, in normal+IPA. fumigatus group, proinflammatory cytokine TNF-α exhibited high expression at the early stages of infection, and the highest expression levels appeared at 48 or 72 h, then decreased and returned to normal level. In the group of the IPA mouse, proinflammatory cytokines TNF-α were released at slow and low level. Persistently low expression of NOD1 and RIP2, was seen in early excessive activation.

Key words: Invasive pulmonary aspergillosis (IPA), nucleotide-binding oligomerization domains (NODs), RIP2, pathogenesis.

INTRODUCTION

Invasive pulmonary aspergillosis (IPA) is an increasingly common opportunistic fungal infection usually occurring in patients with neutropenia and/or corticosteroid exposure. The lungs are involved in about 85% of cases

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of invasive aspergillosis. *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 in hosts with impaired immunity can cause a severe infectious disease called IPA. It is also responsible for some autoimmune diseases, including rheumatoid arthritis, ankylosing spondylitis, inflammatory bowel disease, psoriasis and psoriatic arthritis (Arnold et al., 2009; Tsiodras et al., 2008; Nedel et al., 2009). The mortality rate of IPA has ranged from 60 to 94% (Tomee, 2001; Singh and Paterson, 2005). Hitherto, the pathogenesis of IPA is not clear.

Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) are two major pattern-recognition receptors (PRRs) involved in the early host defense against pathogen invasion (Akira et al., 2006; Li, 2010). Activation of NOD1 by *A. fumigatus* conidia ligand recognition elicited inflammatory cytokines such as TNF-α and IL-10 and stimulated NOD1-induced immune responses (Li, 2010). NOD2 can sense the components of peptidoglycan derived from bacteria, such as muramyl dipeptide (MDP), in the host cytosol. Stimulation of NOD2 by ligand recognition stimulates the NF-κB pathway (Chattoraj et al., 2013; Hasegawa et al., 2008; Nabatov et al., 2013). All these studies showed that NODs play important roles in anti-infectious disease IPA. But there are few studies on the field.

Therefore, we established IPA model of wild type mouse and dynamic investigation of the expression levels of NOD1 and NOD2 mRNA by RT-PCR method, the levels of inflammatory cytokines TNF-α in pulmonary tissues by western blot, with evaluation of the *A. fumigatus* dosage, and the lung pathology; we elucidated the functions of NODs signaling pathway in invasive pulmonary aspergillosis. This study provides 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 3 groups randomly, 10 rats in each group: (1) Normal Group (normal mice); (2) Normal mice with infection (N+ *A. fumigates*); (3) IP A Model Group (normal mice treated with immunosuppressant and inoculated with *A. fumigates*).

**Strain and culture medium**

*A. fumigates* (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. Spores were collected at the concentration of 10⁷/mL and stored at 4°C.

**Main reagents**

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

**IPA model of mice**

According to the literature, the method was as follows: BALB/c mice were injected intraperitoneally with 100 mg·kg⁻¹·d⁻¹ of CY within 2 days. Thereafter, mice were administered intranasally with 50 μL (concentration: 10⁷/mL) spore suspension of *A. fumigates*. In order to maintain the effect of immunosuppression, mice were given additional CY (100 mg·kg⁻¹·d⁻¹) when inoculated with *A. fumigates* at 96h (Luo et al., 2008; Tang et al., 1993).

**Collection and processing of specimen**

Mice with nose inhalation of *A. fumigates* conidia were sacrificed at different time points of 24, 48, 72, 120 and 144 h (2 mice at each time point), then lung tissue was isolated in sterile manner, and conserved in -80°C refrigerator.

**A. fumigates colony counting of lung tissue**

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

**Lung tissue pathology**

Histological injury and spore germination was observed after all the mice produced paraffin sections of lung tissue and conventional HE staining.

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

Primers and PCR reaction conditions are listed in Table 1.

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

First, nuclear protein and total protein were extracted from 100 mg lung tissue; Second, SDS-PAGE electrophoresis was carried out and transferred to semi-dry membrane; once again, it was incubated with the corresponding primary antibody (1:250) and secondary antibodies (1:8000) at 37°C for 1 h; finally, the film was exposed to X-ray after colouring with ECL (a kind of lighting substrate used in west blot detection, Pierce Biotechnology PO Box).

**Statistical methods**

The values of optical density scanning of target band were read which was measured in the agarose gel and X-ray film by image analysis software Bandscan. Afterwards, the expression of its corresponding target gene and protein were respectively
Table 1. Primer sequences and PCR reactive conditions.

<table>
<thead>
<tr>
<th>Aim gene</th>
<th>Primers (5’&gt;3’)</th>
<th>Annealing temperature (°C)</th>
<th>Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Actin</td>
<td>reverse anti-reverse ACGGCCAGGTCATCAGTTAACAGTCCGCTCAGAAGCA</td>
<td>59.3</td>
<td>409</td>
</tr>
<tr>
<td>NOD1</td>
<td>reverse anti-reverse GAACAGGAACATCTGGTCA</td>
<td>67.7</td>
<td>261</td>
</tr>
<tr>
<td>NOD2</td>
<td>reverse anti-reverse AGGATCAGCGGTACATGTC</td>
<td>59.3</td>
<td>438</td>
</tr>
<tr>
<td>RIP2</td>
<td>reverse anti-reverse ATTTGAAGCGGTGCTTTTG</td>
<td>59.3</td>
<td>264</td>
</tr>
</tbody>
</table>

Figure 1. Results of HE staining of the lungs. A–E: N+A. fumigates group: 24, 48, 72, 120, 144 h; F–J: IPA group: 24, 48, 72, 120, 144 h.

Table 2. Results of HE staining of the lungs. A–E: N+A. fumigates group: 24, 48, 72, 120, 144 h; F–J: IPA group: 24, 48, 72, 120, 144 h.

RESULTS

Morphological analysis of lung pathology

There were few lung abscess, hemorrhage and mycelium in lung tissue in the first 48 h after inoculation with A. fumigates; 72 h later, when compared with normal mice, the alveolus space in normal mice with infection enlarged, accompanied with inflammatory responses including inflammatory cell infiltration and hemorrhage injury. As a comparison, IPA group had a lung abscess and severe hemorrhage. In addition, airway epithelial desquamation and mycelium formation were also observed in the IPA group (Figure 1).

Assessment of A. fumigates load in pulmonary tissue

The CFU assay indicated, when compared with normal mice with infection, pulmonary tissue from the IPA group had heavy A. fumigates load (P<0.05) (Figure 2). In contrast, normal mice without A. fumigates inoculation showed negative signal in this assay.

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 𝑥̅ ± s. P<0.05 was used as a standard of significant difference by applying Statistical software SPSS 10.0 to conduct t-test analysis.
**Investigating the expression levels of NOD1 mRNA in mice pulmonary tissue**

The mRNA levels of NOD1 were tested with RT-PCR by time course (Figure 3). Twenty-four hours after inoculation with *A. fumigates*, the expression of NOD1 protein of normal group (N+Af) had a sharp elevation, got to peak at 48 h and then decreased to normal levels after 72 h. IPA mice of NOD1 protein gradually increased after 24 h. It peaked at 72 h, followed by a decline in a level lower than normal mice with infection (*P*<0.05) (Figure 3).

**Investigating the expression levels of NOD2 mRNA in mice pulmonary tissue**

The mRNA levels of NOD2 were tested with RT-PCR by time course (Figure 4). Twenty-four hours after inoculation with *A. fumigates*, the expression of NOD2 protein of normal group (N+Af) had a sharp elevation, got to peak at 48 h and had a little decline, and then kept to high levels after 72 h. IPA mice of NOD2 protein gradually increased after 24 h. It peaked at 48 h, followed by a slowly decline, and finally at 120 h, got back to normal levels of NOD2.
level ($P<0.05$) (Figure 4).

**Investigating the expression levels of RIP2 mRNA in mice pulmonary tissue**

The mRNA levels of RIP2 were tested with RT-PCR by time course (Figure 5). Twenty-four hours after inoculation with *A. fumigates*, the expression of RIP2 protein of normal group (N+Af) had a sharp elevation, got to peak at 48 h. Then had a quick decline, and then got back to normal levels at 72 h; it had another peak at 120 h; and then decline to normal level. IPA mice of RIP2 protein gradually decreased after 24 h; and got to lowest point at 120 h, followed by a sharp increase, and finally got back to normal level ($P<0.05$) (Figure 5).

**Analyzing the expression of TNF-α protein**

Time course experiments were conducted to evaluate the expression of TNF-α protein in mouse pulmonary tissues from different treatment groups. As shown in Figure 6, TNF-α protein levels were measured by western blot. Twenty-four hours after inoculation with *A. fumigates*, the expression of TNF-α protein of normal group (N+Af) gradually increased, and then got to the peak at 72 h; then gradually decline to normal level at 144 h. As a comparison, IPA mice had a lower TNF-α expression with mild alternation (Figure 6).

**Analyzing the expression levels of NOD1, NOD2, RIP2 mRNA and the expression of TNF-α at the same time point for each group**

The mRNA levels of NOD1, NOD2 and RIP2 were compared at the same time point for each group. The expression of NOD1 protein of normal group (N+Af) are higher than normal group at each time course ($P<0.05$). The expression of NOD2 protein of normal group (N+Af)
Figure 6. The dynamic expression of TNF-α protein in different group and the western blotting gram of TNF-α protein at different time point. N: Normal group; N+Af: N+A. fumigates group; IPA: IPA group. A: Normal group; B: N +A. fumigates group; C: IPA group. 1: 24 h; 2: 48 h; 3: 72 h; 4: 120 h; 5: 144 h. ▲ P<0.05, vs at 24 h in N+ A. fumigates group; * P<0.05, vs at 24 h in IPA group.

Figure 7. The expression of NODs and RIP2 mRNA and TNF-α protein at the same time in different groups. N: Normal group; N+Af: N+A. fumigates group; IPA: IPA group. #P<0.05, vs N group; *P<0.05, vs N+ A. fumigates group.

are more than normal groups at 48, 72 and 144 h (P<0.05). RIP2 is higher than normal group at 48 and 72 h after incubation with A. fumigates (P<0.05). When compared with normal group, the expression of NOD1 protein of IPA group is lower at 48, 120 and 144 h (P<0.05); NOD2 are less at all the time point; RIP2 are lower at 24, 48 and 120 h (P<0.05) (Figure 7).

The expression of TNF-α protein in mouse pulmonary tissues was also compared at the same time point for each group. When compared with normal group, TNF-α protein of normal group (N+Af) is higher at all the time point except at 144 h (P<0.05); TNF-α protein of IPA group is lower at 48, 72 and 120 h (P<0.05) (Figure 7D).

DISCUSSION

Since conidia of A. fumigates widely exist in the environment, people usually inhale hundreds of them per day. The inspiratory conidia seldom induce disease in hosts with intact immune system. High risk factors for clinical IPA usually are due to severe neutropenia, long-term antibiotic treatment, steroid therapy, hematopoietic malignancies, organ transplantation, AIDS, autoimmunne diseases, etc. Animal experiments showed: after high dose inoculation with A. fumigates in normal mice, the pathogens would be eliminated within several hours, and the elimination curve was in accordance with first order kinetics; whereas immuno-deficient mice easily suffered from aspergillosis and IPA induced by systemic infection (Schneemann and Schaffner, 1999; Grazzutti et al., 1997; Duong et al., 1998). Thus, healthiness of immune system plays pivotal roles in resistance to A. fumigates infection.

Effective innate immunity is the first line of defense. Phagocytosis of the alveolar 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. fumigates* infection, but also provides specific signals for initiation of adaptive immunity (de Repentigny et al., 1993; Schaffner et al., 1982). However, the mechanism of innate immunity against the infection of *A. fumigates* 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 widely express in macrophages and various cell types (Medzhitov and Janeway, 1997a, b). The NODs family, which was newly discovered, is one of the most important PRRs (Philpott and Girardin, 2004). Most evidence proved that NOD1 and NOD2 play important role in anti-infectious in innate immune response (Boughan et al., 2006; Kobayashi et al., 2005; Chamaillard et al., 2004; Barton et al., 2007). Innate immunity not only confers the first line of defense in resistance to *A. fumigates* infection, but also provides specific signals for initiation of adaptive immunity (Mambula et al., 2002; Kaisho and Akira, 2004). However, the mechanism of innate immunity against the infection of *A. fumigates* is still largely unknown.

Here, in order to systematically mimic patient IPA, the dynamic alternations of NODs, PIR2 and TNF-α cytokines in both normal and immunodeficient mice were evaluated during infection of *A. fumigates*. Cyclophosphamide was used to induce immunosuppression of mice used as model animals. And, the pathological alternation of pulmonary tissues and culture of model animals. Analysis of 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.

NODs 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 NOD1 and NOD2 mRNA between IPA group and normal mice with infection. NODs and RIP2 mRNA in the three groups was slowly increased in the early stage of *A. fumigates* infection. These results indicated that the receptors of NODs and RIP2 were activated after *A. fumigates* inoculation. The expression of NOD2 mRNA in IPA group was higher than normal group in early stage, and then got back to normal level; this indicated that NOD2 may play very important role in *A. fumigates* infection.

Cytokines, an important kind of secretory 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).

We found the expression levels of cytokines (TNF-α) in mouse lung were closely correlated with pulmonary pathological impairment. Normal mice with *A. fumigates* inoculation displayed high levels of proinflammatory cytokines (TNF-α) in the early stage of infection. Their expression levels peaked at 72 h, and thereafter declined to normal level. At the same time, lung pathology results showed obvious hyperemia and hemorrhage appearance before 72 h, and thereafter inflammatory responses were gradually alleviated, which indicated that secretion of proinflammatory cytokines (killing inhalational conidia and preventing the formation of hyphae) was the major inflammatory responses in the early stage of *A. fumigates* infection.

Inflammation is one of the necessary parts of effective immune responses in resistance to IPA. Appropriate inflammatory responses can availably eliminate local *A. fumigates*, 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.

The above results showed immunosuppressed mice with nose inhalation of *A. fumigates* presented pathological alternations similar to clinical IPA cases, indicating the successful establishment of mouse IPA model. Analysis of 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.
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 to 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 (Medvedev et al., 2006).

The results indicate that the NODs signaling pathway in the immunosuppressed mice with A. fumigates inoculation causes the loss of balance between proinflammatory and anti-inflammatory cytokines and eventually leads to the incidence and development of invasive aspergillosis.

Conflict of Interest

The author(s) have not declared any conflict of interests.

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