

## Original Article

# Diversities of interaction of murine macrophages with three strains of *Candida albicans* represented by MyD88, CARD9 gene expressions and ROS, IL-10 and TNF- $\alpha$ secretion

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**Abstract:** Aim: To explore the mechanisms underlying the different responses of macrophages to distinct *Candida albicans* strains. Methods: Bone marrow was collected from mice. Macrophages were independently incubated with 3 *Candida albicans* strains. Results: MyD88 expression in *Candida albicans* 3683 group was significantly higher than that in *Candida albicans* 3630 group and *Candida albicans* SC5314 group, and marked difference was also observed between later two groups ( $P < 0.05$ ). CARD9 expression in *Candida albicans* 3630 group was higher than that in *Candida albicans* 3683 group and *Candida albicans* SC5314 group. Fluorescence intensity was  $46.78 \pm 0.79$  in *Candida albicans* 3630 group,  $32.60 \pm 1.31$  in *Candida albicans* 3683 group and  $19.40 \pm 0.58$  in *Candida albicans* SC5314, and significant difference was observed between any two groups ( $P < 0.05$ ). TNF- $\alpha$  and IL-10 were  $18.9843 \pm 0.7081$  pg/ml and  $11.6690 \pm 0.3167$  pg/ml, respectively, in *Candida albicans* 3683 group, which were markedly higher than those in *Candida albicans* 3630 group and *Candida albicans* SC5314 group ( $P < 0.05$  and  $0.01$ ). Conclusion: Different *Candida albicans* strains may induce CARD9 expression and alter the production of ROS, TNF- $\alpha$  and IL-10 in macrophages, which may be one of mechanisms underlying the different killing effects of macrophages on distinct *Candida albicans* strains.

**Keywords:** *Candida albicans*, macrophage, MyD88, CARD9, IL-10/TNF- $\alpha$ , reactive oxygen species

## Introduction

*Candida albicans* is the most common symbiotic pathogen and conditional pathogenic pathogen, and may cause candidiasis of the skin and mucosa. It may also invade deep tissues and blood, causing diffused systemic candidiasis. Innate immunity is the first line of defense against pathogens. Macrophages are the most important phagocytes in the immune system. Following infection by pathogenic microorganisms, macrophages may recognize the pathogen-associated molecular pattern on the microorganisms via the pattern recognition receptor (PRRs), initiating the immune response to these pathogenic microorganisms. Studies have revealed that PRRs mainly include Toll-like receptors (TLRs) on cell membrane and C-type

lectin receptors (CLRs) [1]. TLRs may activate transcription factors NF- $\kappa$ B and P38MAPK in a MyD88 dependent manner [2]. Dectin-1 and dectin-2 may activate p38, Erk, JNK and NF- $\kappa$ B [3] via Syk-CARD9 signaling pathway. These then promote the synthesis of IL-2, IL-6, TNF- $\alpha$  and IL-23, facilitate the maturation and transformation of dendritic cells (DCs) into functional antigen presenting cells (APCs) [4], and enhance the killing effect of neutrophils on the *Candida albicans* [5]. Interaction between Dectin-1 and TLR2 enhances the TLR induced NF- $\kappa$ B activation and increase the synthesis of IL-12 and TNF- $\alpha$  and the production of reactive oxygen species (ROS) [6].

It has been confirmed that ROS not only is a group of toxic molecules but has high biological

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**Table 1.** mRNA expression of MyD88 and CARD9 in macrophages after treatment with different *Candida albicans* stains for 30 min

Group	MyD88	CARD9
3630 group	0.00042±0.00028*	0.00031±0.00012#
3683 group	0.00071±0.00039*	0.00009±0.00008
SC5314 group	0.00016±0.00006*	0.00005±0.00003

Footnotes: \* $P < 0.05$  among three groups; # $P < 0.05$  vs SC5314 group and 3683 group.

**Table 2.** mRNA expression of MyD88 and CARD9 in macrophages after treatment with different *Candida albicans* stains for 1 h

Group	MyD88	CARD9
3630 group	0.00115±0.00126	0.00031±0.00025
3683 group	0.00089±0.00122	0.00061±0.00038
SC5314 group	0.00027±0.00323	0.00019±0.00023

Footnotes: no significant difference was observed among three groups.

**Table 3.** mRNA expression of MyD88 and CARD9 in macrophages after treatment with different *Candida albicans* stains for 2 h

Group	MyD88	CARD9
3630 group	0.00067±0.00008	0.00013±0.00001
3683 group	0.08444±0.10324	0.00015±0.00007
SC5314 group	0.00253±0.00435	0.00048±0.00012#

Footnotes: # $P < 0.01$  vs. 3630 group and 3683 group.

**Table 4.** mRNA expression of MyD88 and CARD9 in macrophages after treatment with different *Candida albicans* stains for 4 h

Group	MyD88	CARD9
3630 group	0.00992±0.01807	0.00015±0.00006
3683 group	0.01394±0.02170	0.00053±0.00020#
SC5314 group	0.00230±0.00310	0.00009±0.00010

Footnotes: # $P < 0.01$  vs. SC5314 group and 3630 group.

activities and is involved in a variety of cytokine-mediated biological effects [7]. There is evidence showing that the interaction between dectin-1/CARD9 signaling pathway and TLR2/MyD88 signaling pathway may enhance the TLR mediated NF- $\kappa$ B activation and promote the synthesis of cytokines IL-12 and TNF- $\alpha$  and the production of ROS [6]. Wu et al found CARD9 was an important factor involved in the suppression of fungal and bacterial infection by

macrophages and the interaction between CARD9 and LyGD1 may stimulate the production of ROS.

TNF- $\alpha$  is a cytokine secreted by macrophages and activated T cells and has multiple biological activities. IL-10 acts mainly to inhibit the antigen processing of macrophages and suppress the production of pro-inflammatory cytokines. Studies have indicated that TLRs mainly activate transcription factors NF- $\kappa$ B and P38MAPK in a MyD88 dependent manner, which promote the secretion of important pro-inflammatory cytokines (such as TNF- $\alpha$ ) and anti-inflammatory cytokines (such as IL-10). However, the activation of Dectin-1/2-Syk-CARD9 signaling pathway mainly facilitates the secretion of pro-inflammatory cytokines (such as IL-6, IL-23, TNF- $\alpha$  and IL-12) and may induce the production of IL-17 by helper T cells.

Our previous study showed macrophages from mice had the most potent killing effect on *Candida albicans* 3683 and the weakest ability to kill *Candida albicans* SC5314, and the killing effect on *Candida albicans* 3630 was between above two effects [8, 9]. Thus, it is imperative to elucidate the difference in the killing effects of macrophages on distinct *Candida albicans* strains.

### Materials and methods

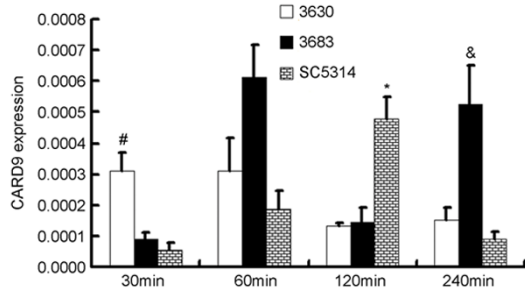
#### Materials

Three *Candida albicans* stains (SC5314, 3630, 3683) were kindly provided by the School of Dentistry of University of Queensland in Australia. RPMI1640, fetal bovine serum (Gibco, USA), C57/6J mouse, mouse L929 fibroblasts (Experimental Animal Center of Sun Yat-sen Universtiy), reverse transcription kit (Ferments, USA), Trizol, RT-PCR kit (Invitrogen, USA) and automatic fluorescence quantitative PCR (ABI 7500) were used in the present study.

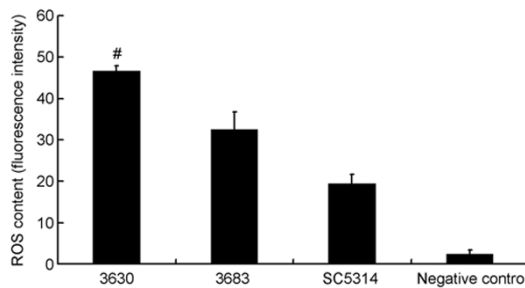
#### Coculture of macrophages and different *Candida albicans* strains

1) Macrophage colony-stimulating factor was harvested from the supernatant of mouse fibroblast line (L929 cells) and used to treat macrophages from mouse bone marrow in vitro. Flow

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**Figure 1.** CARD9 expression in macrophages treated with different *Candida albicans* stains for 120 min (SC5314 group >3683 group >3630 group) and 240 min (3683 group >3630 group >SC5314 group). # $P < 0.05$  vs. SC5314 group and 3683 group at 30 min, \* $P < 0.01$  vs. 3630 group and 3683 group at 2 h, & $P < 0.01$  vs. SC5314 group and 3630 group.



**Figure 2.** ROS content in macrophages after incubation with different *Candida albicans* stains for 30 min. # $P < 0.05$  vs. any other group.

cytometry was done to detect the surface markers of macrophages (F4/80). After induction and differentiation, the purity of macrophages was higher than 94% and these macrophages were used in following experiments.

2) Macrophages were seeded into 6-well plates ( $1 \times 10^6$ /well). Cells were grown in 1 mL of RPMI1640 containing 1% antibiotics in an environment of 5% CO<sub>2</sub> at 37°C overnight. Three *Candida albicans* stains (3630, 3683 and SC5314) were seeded into fresh Sandcastle's medium at 37°C for 24 h. Then, the *Candida albicans* stains were collected and washed with PBS thrice by centrifugation. The *Candida albicans* density was adjusted to  $1 \times 10^7$ /mL.

3) Macrophages were divided into SC5314 group, 3630 group and 3683 group in which macrophages were incubated with *Candida albicans* SC5314, *Candida albicans* 3630 and *Candida albicans* 3683, respectively. In nega-

**Table 5.** ROS content in macrophages treated with different *Candida albicans* stains for 30 min

Group	ROS (mean fluorescence intensity)
SC5314 group	19.40±0.58
3630 group	46.78±0.79#
3683 group	32.60±1.31
Control group	2.52±0.66

Footnotes: # $P < 0.05$  vs. any other group.

tive control group, macrophages were grown alone; in positive control group, macrophages were treated with 1.0 µg/mL LPS.

4) Macrophages were counted and incubated overnight. The medium was removed, and macrophages were treated with *Candida albicans* or LPS for 30 min, 1 h, 2 h, and 4 h. Then, cells were harvested, treated with Trizol and stored at -80°C.

### Detection of MyD88 and CARD9 mRNA expression by fluorescence quantitative PCR

1) Primers: mRNA of target genes was obtained from Gene Bank, according to which primers were designed as follows: MyD88: forward: 5'-GATGCGGAGCCAGATTCTCT-3'; reverse: 5'-TC-ATCATTGAACACGGGTTGA-3'; CARD9: forward: 5'-AGTCCTGAACCCCGATGATG-3'; 5'-CAGGAGC-ACACCCACTTTCC-3'; GAPDH: forward: 5'-CCAC-CCAGAAGACTGTGGAT-3'; reverse: 5'-GGA TGCA-GGGATGATGTTCT-3'

2) Quantitative PCR: Samples and positive standard were independently used to prepare following mixture: 5× SYBR Green I PCR buffer (10 µL), 10 pmol/µL forward primer (1 µL), 10 pmol/µL reverse primer (1 µL), 10 mM dNTPs (1 µL), 3 U/µL Taq polymerase (1 µL), cDNA or positive standard (1 µL) and ddH<sub>2</sub>O (35 µL).

Buffer used for PCR included 10 mM Tris-HCl (pH8.0), 50 mM KCl and 2 mM MgCl<sub>2</sub>. Reaction was performed at 93°C for 3 min, and then 40 cycles of 93°C for 30 sec and 55°C for 45 sec. Data were automatically calculated after reaction and expressed as B (copies/µL cDNA). The concentration of total RNA was different among samples, and the relative expression of target gene was normalized to that of an internal reference (GAPDH) as follows: A = B1 (target gene)/B2 (GAPDH)

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**Table 6.** TNF- $\alpha$  content in macrophages after incubation with different *Candida albicans* stains

Group	8 h (pg/mL)	12 h (pg/mL)	24 h (pg/mL)
SC5314 group	14.1682 $\pm$ 0.3087	13.9901 $\pm$ 0.1694	14.2443 $\pm$ 0.2852
3630 group	14.1748 $\pm$ 0.3006	14.2284 $\pm$ 0.2841	15.5143 $\pm$ 0.2517*
3683 group	18.9843 $\pm$ 0.7081*	14.6215 $\pm$ 0.4032	14.5665 $\pm$ 0.4497
Control group	-	-	-

Footnotes: \* $P < 0.01$  vs. any other group at 8 h;  $P > 0.05$  among group at 12 h;  $P < 0.01$  vs. any other group at 24 h.

**Table 7.** IL-10 content at different time points in macrophages treated with different *Candida albicans* strains

Group	8 h (pg/mL)	12 h (pg/mL)	24 h (pg/mL)
SC5314 group	3.2967 $\pm$ 0.0239#	-	-
3630 group	0.4896 $\pm$ 0.0068	-	-
3683 group	11.6690 $\pm$ 0.3167*	3.5380 $\pm$ 0.2808*	0.3931 $\pm$ 0.0039*
Control group	-	-	-

Footnotes: # $P < 0.01$  vs. 3630 group at 8 h, \* $P < 0.01$  vs. 8 h, 12 h or 24 h in 3683 group.

### Detection of ROS by flow cytometry

1) Grouping: Macrophages were divided into SC5314 group, 3630 group and 3683 group in which macrophages were incubated with *Candida albicans* SC5314, *Candida albicans* 3630 and *Candida albicans* 3683, respectively. In negative control group, macrophages were grown alone. 2) Co-culture: Macrophages were counted and washed with PBS by centrifugation. The medium was removed and macrophages were incubated with different *Candida albicans* strains at a ratio of 1:10 and 0.5 mL of 20  $\mu$ M DCFH-DA at 37°C for 30 min. After washing in PBS twice, macrophages were re-suspended in 0.5 ml of PBS. Flow cytometry (FCM) was done to detect the green fluorescence to evaluate the content of ROS.

### Detection of cytokines

1) Coculture: Macrophages were counted and incubated overnight. The medium was removed and macrophages were incubated with different *Candida albicans* strains at a ratio of 1:1. The final volume of resultant mixture was adjusted to 1 ml with 1640 medium.

2) Macrophages were incubated for 8 h, 12 h and 24 h and the supernatant was harvested and stored at -20°C.

3) The concentration of IL-10/TNF- $\alpha$  in the supernatant was detected with ELISA kits.

### Statistical analysis

Data were expressed as mean  $\pm$  standard deviation ( $\bar{x} \pm s$ ) and statistical analysis was performed with SPSS version 11.0. Data between two groups were compared with independent sample t test, and those among different groups with one way analysis of variance. Homogeneity of variance test was done firstly. Data with homogeneity of variance were compared with Bonferroni test between groups. Data without homo-

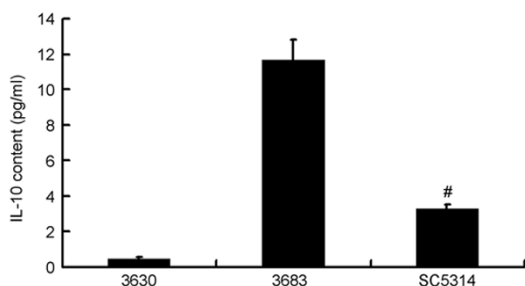
geneity of variance were compared with Rank sum test between groups. A value of  $P < 0.05$  was considered statistically significant.

## Results

### mRNA expression of MyD88 and CARD9 in macrophages after incubation with different *Candida albicans* strains

The mRNA expression of MyD88 and CARD9 was undetectable at different time points in macrophages without incubation with *Candida albicans*. After incubation with *Candida albicans* for 30 min, 1 h, 2 h and 4 h, the mRNA expression of both MyD88 and CARD9 was detectable in macrophages. This suggests that incubation with *Candida albicans* may activate the signaling pathways in macrophages. When the macrophages were incubated with different *Candida albicans* stains for 30 min (**Table 1**), the MyD88 expression was 0.00071 $\pm$ 0.00039 in 3683 group, which was significantly higher than that in 3630 group (0.00042 $\pm$ 0.00028) and SC5314 group (0.00016 $\pm$ 0.00006), and significant difference was also noted between later two groups ( $P < 0.05$ ). The CARD9 expression was 0.00031 $\pm$ 0.00012 in 3630 group, which was dramatically higher than that in 3683 group (0.00019 $\pm$ 0.00008) and SC5314 group (0.00015 $\pm$ 0.00003) ( $P < 0.05$ ). This sug-

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**Figure 3.** IL-10 content in macrophages treated with different *Candida albicans* strains. # $P < 0.01$  vs. 3630 group at 8 h.

gests that incubation with *Candida albicans* may activate both MyD88 and CARD9 signaling pathways, but the extent of activation is different between two pathways. At 1 h, 2 h and 4 h after incubation, the MyD88 expression was comparable among three groups ( $P > 0.05$ ) (Tables 2-4). At 1 h, CARD9 expression was comparable among three groups ( $P > 0.05$ ) (Table 2). At 2 h, CARD9 expression was  $0.00048 \pm 0.00012$  in SC5314 group, which was significantly different from that in other two groups ( $P < 0.01$ ) (Table 3). At 4 h, CARD9 expression in 3683 group was  $0.00053 \pm 0.00020$  in 3683 group, which was markedly different from that in other two groups ( $P < 0.01$ ) (Table 4). This suggests that the MyD88 expression tends to be comparable among three groups over time, and difference was only observed in the CARD9 expression among these groups (Figure 1). These findings indicate that the extent of activation of signaling pathway involving CARD9 is significantly different among three groups, which might be one of mechanisms underlying the difference in the killing effect of macrophages after treatment with distinct *Candida albicans* stains.

### Detection of ROS by flow cytometry

Macrophages were treated with different *Candida albicans* stains for 30 min, and flow cytometry was performed to detect ROS. Results showed the ROS content was the highest in 3630 group and the lowest in control group, and the ROS content in 3683 group was also significantly higher than that in SC5314 group. Statistical analysis showed marked difference was observed in the ROS content between any two groups ( $P < 0.05$ ) (Table 5 and Figure 2).

### TNF- $\alpha$ content in macrophages after incubation with different *Candida albicans* stains

Statistical analysis (Table 6) showed TNF- $\alpha$  content was extremely low and undetectable in macrophages from mouse bone marrow without treatment with *Candida albicans*. At 8 h, the TNF- $\alpha$  content was the highest in 3683 group ( $18.9843 \pm 0.7081$  pg/mL) and significantly higher than that in SC5314 group and 3630 group. At 12 h, the TNF- $\alpha$  content was comparable among groups. At 24 h, the TNF- $\alpha$  content in 3630 group was markedly higher than that in 3683 group and SC5314 group. When compared with control group, the TNF- $\alpha$  content increased dramatically in macrophages at 8 h, 12 h and 24 h after incubation with *Candida albicans*, suggesting that *Candida albicans* may stimulate macrophages to secrete TNF- $\alpha$ . In 3683 group, the TNF- $\alpha$  content reduced significantly over time ( $P < 0.01$ ). In 3630 group, the TNF- $\alpha$  content increased markedly over time ( $P < 0.01$ ). However, in SC5314 group, the TNF- $\alpha$  content remained unchanged over time.

### IL-10 content in macrophages after incubation with different *Candida albicans* stains

IL-10 content was the highest in macrophages treated with *Candida albicans* 3683, and significant difference was also observed between SC5314 group and 3630 group ( $P < 0.01$ ) (Table 7). The IL-10 content was extremely low over time and undetectable in 3630 group and SC5314 group. However, in 3683 group, the IL-10 content reduced significantly over time ( $P < 0.01$ ; Table 7 and Figure 3).

## Discussion

Macrophages are important phagocytes in the immune system. Following infection by pathogenic microorganisms, macrophages can recognize the PAMPs on these microorganisms via PRR to initiate the immune response [1, 2] aiming to remove antigens. Typical PAMPs are components of bacterial wall (such as LPS and teichoic acid). The interaction between PRR and PAMPs may induce signal cascade and expression of gene involved in immune response, to activate the biological defense against microorganisms. Of PRRs, TLR family is extensively studied. There are 13 members in TLR family. TLR2 is a receptor of teichoic acid on Gram

positive bacteria and TLR4 is a receptor of LPS of Gram negative bacteria. TLR9 can bind to the DNA of Gram positive and negative bacteria. Actually, all the pathogenic bacteria can be recognized by one or more TLR [10, 11].

MyD88 is one of adapter molecules involved in the signal transduction via the intracellular domain of TLR. Almost all the TLRs exert effect in a MyD88 dependent manner. MyD88 may free NF- $\kappa$ B via phosphorylation, and then NF- $\kappa$ B translocates into the nucleus and binds to DNA exerting regulatory effect, which may regulate the production of pro-inflammatory cytokines and adhesion molecules [11, 12]. Following stimulation by PAMPs, TNF- $\alpha$  is one of important effector molecules that activate NF- $\kappa$ B in a TLR-MyD88 dependent manner. This may promote the killing effect on bacteria and the phagocytic activity, and also plays an important role in the inflammatory response [13-15]. In TLR mediated cascade, the cytoplasmic domain of TLR interacts with adapter protein MyD88 to phosphorylate I- $\kappa$ B, resulting in the I- $\kappa$ B degradation. Then, the activated NF- $\kappa$ B translocates into the nucleus to activate the expression of genes involved in immune response. Thus, MyD88 play an important role in the initiation of NF- $\kappa$ B dependent signal cascade. However, as a transcription factor, NF- $\kappa$ B plays crucial roles in both innate immunity and adaptive immunity. NF- $\kappa$ B can regulate the expression of multiple pro-inflammatory cytokines and immune effector proteins. MyD88 may activate NF- $\kappa$ B and AP-1 in RAW264 cells and promote the production of IFN- $\alpha$ , TNF- $\alpha$  and IL-12 in DCs from bone marrow [16, 17].

CARD9 a new ligand identified in recent years. CARD9 is a key signal molecule in the CLR mediated signal transduction. Increasing studies reveal that CARD9 play vital roles in the innate immunity. CARD9 may induce some cellular responses, including ROS production, activation of PLA2 and COX2 and regulation of production of some cytokines and chemokines (such as IL-10) [1, 3]. Although CARD9 has little effect in the MyD88-TLR mediated immune response, CARD9 can mediate the activation of myeloid cells and the production of cytokines, which are involved in the innate immunity against fungi [18]. Deficiency of CARD9 gene encoding BCL10 may cause the impairment of ITAM mediated activation of NF- $\kappa$ B in myeloid cells [19, 20].

In our study, different downstream signaling pathways were activated at different time points in macrophages after treated with *Candida albicans*. The low MyD88 expression tended to be comparable among three groups in the early stage, but when co-cultured with *Candida albicans* stains for 120 min, the MyD88 expression increased for 10-100 times, especially in the 3683 group. This suggests that CARD9 mediated signaling pathway is activated to different extents in macrophages treated with *Candida albicans* stains and may be one of key molecules involved in the killing effect of macrophages on the *Candida albicans*. The difference in the CARD9 expression might be associated with the expression and distribution of PAMPs of the *Candida albicans*. PAMPs are closely related to the pathogenicity of *Candida albicans* and include phospholipomannan (PLM), LPS,  $\beta$ -1,3 glucan and  $\beta$ -1, 6 glucan. However, PAMPs are different among distinct *Candida albicans* stains, resulting in the different affinity to PRRs and activation of different signaling pathway. It has been found that CLRs play important role in the anti-fungal infection. CLRs can mediate the binding to, uptake and killing of fungi and may help the initiation or regulation of immune response. Taken together, the CLR-CARD9 signaling pathway may play a more important role in the protection against *Candida albicans* infection when compared with TLRs-MyD88 signaling pathway.

ROS includes superoxide radicals, hydrogen peroxide, downstream peroxides and hydroxides. Studies have shown that ROS is involved in multiple physiological and pathological processes related to cell growth, proliferation, development, differentiation, aging and apoptosis. In addition, ROS is also a group of important intracellular mediators and plays important role in mediating the differentiation of macrophages [21, 22]. Moreover, ROS may up-regulate the expression of inflammatory cytokines and mediators via complex signaling pathway, further aggregating inflammatory response.

In the present study, reactive oxygen species assay kit was used to detect ROS in macrophages with DCFH-DA as a probe. DCFH-DA is the most common and sensitive probe used to detect the intracellular ROS. After incubation with different *Candida albicans* strains for 30

min, the mean fluorescence intensity was the highest in 3630 group. CARD9 is an important signaling pathway involved in the ROS production. ROS is produced in macrophages soon after incubation with *Candida albicans* and plays an important role in anti-fungal infection. In our study, flow cytometry was performed to detect ROS after incubation with *Candida albicans* for 30 min. Results showed ROS content was the highest in 3630 group which was consistent with the change in CARD9 in PCR. This suggests that CARD9 is a key signal molecule involved in the production of ROS.

After fungal infection, the IL-10 content in the serum or the infected site is significantly higher than that in healthy subjects [23, 24]. Animal studies also reveal that IL-10 deficient mice are more resistant to *Candida albicans* and *aspergillus* [25], suggesting that the production of Th2-type cytokines is associated with the susceptibility to fungal infection [26, 27].

Activation of MyD88 induces the production of IL-10 and TNF- $\alpha$ , but activation of CARD9 promotes the IL-10 synthesis. IL-10 is a representative one of Th2-type cytokines and has potent inhibitory effect on monocytes/macrophages. IL-10 may inhibit the anti-bacterial effect of monocytes/macrophages and suppress the production of pro-inflammatory cytokines (such as TNF- $\alpha$ ) to compromise inflammatory response. TNF- $\alpha$  is a representative one of Th1 type cytokines. TNF- $\alpha$  is an intermediate produced by monocytes/macrophages after exogenous stimulation (such as microorganisms and endotoxin) and can regulate specific immune response and acute inflammatory response. TNF- $\alpha$  may activate neutrophils and monocytes/macrophages, promote the differentiation of Th0 cells into Th1 cells, enhance the activity of Th1 cells, effectively inhibit the differentiation of Th0 cells into Th2 cells and improve the killing effect of monocytes/macrophages. The balance between pro-inflammatory cytokines and anti-inflammatory cytokines is a basis for the maintenance of normal immune status. Our results showed macrophages after incubation with different *Candida albicans* strains could secrete 2 cytokines, the concentration of which changed over time. Three *Candida albicans* strains may induce the macrophages to produce TNF- $\alpha$  and IL-10, and TNF- $\alpha$  synthesis continued, but IL-10 secretion was gradually inhibited over time. This suggests that the acti-

vation of MyD88 and CARD9 as important molecules is dependent on time, which leads to the difference in the expression of TNF- $\alpha$  and IL-10. Soon after incubation with *Candida albicans*, both MyD88 and CARD9 are expressed, and thereafter the activation of CARD9 signaling pathway increases TNF- $\alpha$  expression and inhibits IL-10 expression, leading to continuous expression of TNF- $\alpha$ , which may increase the phagocytic activity and killing effect of macrophages, resulting in the difference in the killing effect of macrophages on distinct *Candida albicans* strains.

Our previous study showed mouse macrophages had the most potent killing effect on *Candida albicans* 3683 and the weakest killing effect on *Candida albicans* SC5314, and that on *Candida albicans* 3630 was between them. This suggests that there is difference in the killing effect of macrophages on distinct *Candida albicans* strains. We speculate that this difference is closely associated with the recognition of PRRs on macrophages to PAMP on *Candida albicans* and the resultant immune response. Our results confirmed the difference in the killing effect of macrophages on *Candida albicans* and we further detected the mRNA expression of MyD88 and CARD9 in macrophages treated with different *Candida albicans* strains. Results showed the downstream signaling pathways were different at different time points in macrophages incubated with different *Candida albicans* strains. The extent of CARD9 activation was significantly different among three groups. Thus, CARD9 may be one of key signal molecules involved in the difference in the killing effect of macrophages on *Candida albicans*. To confirm this hypothesis, we performed flow cytometry to detect ROS in macrophages soon after incubation with *Candida albicans*. Results indicated CARD9 was an important molecule involved in the ROS production and played an important role in the secretion of ROS by macrophages soon after *Candida albicans* infection. After incubation with *Candida albicans* for 30 min, the CARD9 expression was the highest in 3630 group, which was consistent with results from PCR, and further confirmed that CARD9 was a key molecule involved in ROS production. In addition, all the *Candida albicans* strains stimulated macrophages to produce TNF- $\alpha$  and IL-10, but IL-10 production was significantly inhibited at late stage. This implies that the difference in the extent of activation of

CARD9 related signaling pathway is dependent on time and results in difference in the expression of TNF- $\alpha$  and IL-10.

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#### Disclosure of conflict of interest

None.

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