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***Malassezia globosa* activates NLRP3 inflammasome in human sebocytes**

Wei Li<sup>1</sup>, Tian Wang<sup>1</sup>, Wen-Bin Huang<sup>1</sup>, Fang-Gu Li<sup>1</sup>, Juan-Hua Quan<sup>2</sup>, Ge Shi<sup>1,3</sup>,  
Christos C. Zouboulis<sup>4</sup>, and Yi-Ming Fan<sup>1</sup>

<sup>1</sup> Department of Dermatology, Affiliated Hospital of Guangdong Medical University,  
Zhanjiang, Guangdong, China

<sup>2</sup> Department of Gastroenterology, Affiliated Hospital of Guangdong Medical  
University, Zhanjiang, Guangdong, China

<sup>3</sup> Department of Dermatology, Sixth Affiliated Hospital, Sun Yat-sen University,  
Guangzhou, Guangdong, China

<sup>4</sup> Departments of Dermatology, Venereology, Allergology and Immunology, Dessau  
Medical Center, Brandenburg Medical School Theodor Fontane and Faculty of Health  
Sciences Brandenburg, Dessau, Germany

Correspondence: Yi-Ming Fan, Department of Dermatology, Affiliated Hospital of  
Guangdong Medical University, 57 Southern Renmin Avenue, Xiashan District,  
Zhanjiang, Guangdong, 524001, China. E-mail: [ymfan1963@163.com](mailto:ymfan1963@163.com)

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Phone: 86-759-2369983

Fax: 86-759-2231754

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Sebocytes can serve as immunocompetent cells and contribute to cutaneous inflammation (Picardo et al., 2015; Zouboulis et al., 2020). NLRP3 inflammasome is a cytosolic multiprotein complex composed of NLRP3, ASC, and pro-caspase-1. Upon detection of pathogen/damage-associated molecular patterns, it is assembled to activate caspase-1, trigger maturation and release of IL-1 $\beta$ , IL-18, gasdermin-D, and induce pyroptosis (Moretti and Blander, 2021). Different *Malassezia* species induce NLRP3 inflammasome activation and subsequent IL-1 $\beta$  secretion in keratinocytes (Park et al., 2021), macrophages and dendritic cells (DCs; Kistowska et al., 2014; Wolf et al., 2021), but it is unknown whether they can activate NLRP3 inflammasome in sebocytes. Considering *Propionibacterium acnes* activated NLRP3 inflammasome in SZ95 sebocytes (Li et al., 2014), we postulated the same role of *Malassezia* in sebocytes and then investigated activation mechanism of *M. globosa*-induced NLRP3 inflammasome in SZ95 sebocytes.

Immunohistochemistry showed overexpression of Toll-like receptor (TLR)2/4, Dectin-1, NLRP3 and IL-1 $\beta$  but not Dectin-2 in sebaceous glands of *Malassezia* folliculitis lesions compared with normal skin (**Supplementary Figure S1a**), suggesting a possible role of TLR2/4, Dectin-1 and NLRP3 inflammasome activation of sebaceous glands in *Malassezia* folliculitis pathogenesis. Transmission electron microscopy visualized endocytic spores with intact morphology and serrated inner wall in SZ95 sebocytes infected with 40 multiplicity of infection (MOI) *M. globosa* for 12 h and 24 h (**Supplementary Figure S1b**).

To screen the optimal MOI and time, SZ95 sebocytes were infected with *M. globosa*

at 0-40 MOIs for 6-72 h. Enzyme-linked immunosorbent assay (ELISA) revealed that supernatant IL-1 $\beta$  levels were incremental at 20-40 MOI ( $P<0.001-0.01$ ) for 24 h (**Figure 1a**). CCK-8 assay displayed remarkably decreased viability of SZ95 sebocytes at 40 MOI for 24 h ( $P<0.01$ ) (**Figure 1b**). In addition, quantitative real-time PCR (qRT-PCR) and ELISA showed a time-dependently up-regulated IL-1 $\beta$  expression in SZ95 sebocytes infected with 30 MOI *M. globosa* during 24 h, which topped at 24 h ( $P<0.01-0.05$ ) (**Figure 1c-d**). Therefore, 30 MOI *M. globosa* infection for 24 h was chosen for subsequent experiment.

The gene expression of 23 known human NOD-like receptors (NLRs), AIM2, pyrin, ASC, caspase-1/4/5/8, gasdermin-D, IL-1 $\beta$ , and IL-18 was examined in SZ95 sebocytes infected with 30 MOI *M. globosa* for 72 h (**Supplementary Table S1**). *NLRP3*, *ASC*, *CASP1*, *CASP4*, *IL1B*, and *gasdermin-D* mRNAs were up-regulated at 72 h ( $P<0.05$ ), while *NOD1*, *NOD2*, *NLRP1*, *NLRP2*, *NLRP14*, *NLRPX*, *NLRC5*, *APAF-1*, *CIITA*, *NAIP*, and *IL18* mRNAs were down-regulated at 72 h ( $P<0.05$ ). Western blot (WB) found overexpression of NLRP3, ASC, caspase-1, and IL-1 $\beta$  at 72 h, of which mature IL-1 $\beta$  peaked at 6 h ( $P<0.001$ ), NLRP3 and caspase-1 at 12 h ( $P<0.001$ ), and ASC and IL-1 $\beta$  at 24 h ( $P<0.001-0.05$ ) (**Figure 1e**). These results suggest that *M. globosa* could mainly trigger NLRP3 inflammasome. In addition, SZ95 sebocytes were transfected with NLRP3, ASC, caspase-1, or scramble siRNAs for 24 h, and then infected with *M. globosa* for 24 h. qRT-PCR, WB and ELISA revealed downregulation of NLRP3, ASC, caspase-1, and IL-1 $\beta$  in SZ95 sebocytes following *NLRP3*, *ASC* and *caspase-1* silencing ( $P<0.001-0.01$ ) (**Figure 1f-h**), confirming that *M. globosa*-induced IL-1 $\beta$

release is mediated by NLRP3 inflammasome in SZ95 sebocytes.

The canonical NLRP3 inflammasome activation normally requires two signals. The first/priming signal occurs mainly through TLR/MyD88 and results in NF- $\kappa$ B activation and subsequent upregulation of NLRP3 and pro-IL-1 $\beta$  (Mathur et al., 2018; Moretti and Blander, 2021). Syk signaling is necessary for NLRP3 inflammasome activation by *Malassezia* in human and mouse DCs (Kistowska M, 2014; Wolf et al., 2021). So the role of TLR 2/4 and Dectin-1/2 in NLRP3 inflammasome activation was studied in SZ95 sebocytes. qRT-PCR and WB showed that their expression increased with infection time ( $P<0.001-0.05$ ) (**Supplementary Figure S2a, Supplementary Table S1**). Immunofluorescence stain demonstrated increased intensity of TLR2, TLR4, Dectin-1, and Dectin-2 in cytomembrane and cytoplasm after infected with *M. globosa* or treated with TLR2 agonist Pam3Cys-Ser-(Lys)<sub>4</sub>, TLR4 agonist lipopolysaccharide (LPS), Dectin-1 agonist Zymosan, and Dectin-2 agonist Mannan for 24 h ( $P<0.001-0.01$ ) (**Supplementary Figure S3**), suggesting that *M. globosa* can induce TLR2, TLR4, Dectin-1 and Dectin-2 expression in SZ95 sebocytes. Furthermore, TLR2 inhibitor C29, Dectin-1 inhibitor laminaran or TLR2/Dectin-1 silencing restrained NLRP3, ASC, caspase-1, and IL-1 $\beta$  expression ( $P<0.001-0.05$ ) (**Supplementary Figure S2b-e**), indicating that TLR2 and Dectin-1 **contribute to** *M. globosa*-activated NLRP3 inflammasome in SZ95 sebocytes.

The second signal mechanisms of NLRP3 inflammasome activation include, among others, K<sup>+</sup> efflux, reactive oxygen species (ROS), lysosomal disruption, mitochondrial damage, NLRP3 mitochondrial translocation, and Ca<sup>2+</sup> influx (Mathur et al., 2018;

Moretti and Blander, 2021). Various fungal species can trigger canonical NLRP3 inflammasome through ROS, K<sup>+</sup> efflux, and lysosomal cathepsins release (Tang et al., 2018). NLRP3 inflammasome activation by *Malassezia* was dependent on K<sup>+</sup> efflux and phagocytosis in mouse DCs (Wolf et al., 2021). Proteases stimulate ROS release from various cells (Li et al., 2014). To understand NLRP3 inflammasome activation mechanisms, K<sup>+</sup>, ROS, and mitochondrial membrane potential (MtMP) were detected using flow cytometry in SZ95 sebocytes infected with 10-30 MOI *M. globosa* for 24 h. We found the decreased K<sup>+</sup> and MtMP, and increased ROS and mitochondrial ROS in 20 and 30 MOI groups ( $P<0.001-0.05$ ) (**Figure 2a-d**). NLRP3, caspase-1, and IL-1 $\beta$  levels were down-regulated in SZ95 sebocytes by pretreatment with metalloprotease inhibitor ethylene diamine tetraacetic acid (EDTA), KCl, ROS inhibitor N-acetyl-L-cysteine (NAC), ATP-sensitive K<sup>+</sup> channel inhibitor glibenclamide, and phagocytosis inhibitor cytochalasin D ( $P<0.001-0.05$ ) (**Figure 2e-f**). Moreover, laser confocal microscopy displayed NLRP3 and ASC mitochondrial translocation after *M. globosa* infection (**Figure 2g-h**). These results suggest that secreted proteases, phagocytosis, K<sup>+</sup> efflux, ROS, mitochondrial damage, and NLRP3/ASC mitochondrial translocation are implicated in *M. globosa*-induced NLRP3 inflammasome activation in SZ95 sebocytes.

*Nlrp3*-deficient mice were used to evaluate *in vivo* role of NLRP3 inflammasome in *M. globosa*-induced skin infection. Compared with wild-type mice, IL-1 $\beta$  expression and lymphocytic infiltrate decreased in *Nlrp3*<sup>-/-</sup> mice ( $P<0.001-0.05$ ) (**Supplementary Figure S4**), implying that *M. globosa*-induced skin inflammation could be at least partly mediated through NLRP3 inflammasome activation in sebaceous glands.

In conclusion, this study demonstrates that *M. globosa* can activate NLRP3 inflammasome and subsequent IL-1 $\beta$  release in SZ95 sebocytes via two signals (**Supplementary Figure S5**). Its impact on pilosebaceous inflammation is worthy of further investigation.

### **Data availability statement**

No datasets were generated or analyzed during the current study. All data in this study are included in this published article and its Supplementary Material files.

### **ORCIDiS**

Wei Li: <https://orcid.org/0000-0001-5278-5936>

Tian Wang: <https://orcid.org/0000-0001-8004-6006>

Wen-Bin Huang: <https://orcid.org/0000-0001-9418-8099>

Fang-Gu Li: <https://orcid.org/0000-0002-1003-623X>

Juan-Hua Quan: <https://orcid.org/0000-0003-4731-3410>

Ge Shi: <https://orcid.org/0000-0002-1072-2213>

Christos C. Zouboulis: <http://orcid.org/0000-0003-1646-2608>

Yi-Ming Fan: <https://orcid.org/0000-0002-6206-1306>

### **Conflict of Interest**

The authors state no conflict of interest.

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### **Author Contributions**

Conceptualization: YMF, WL, TW; Funding Acquisition: YMF; Investigation: WL, TW, WBH, FGL, JHQ; Methodology: WL, TW, WBH, FGL, JHQ; Project Administration: YMF, GS, JHQ; Resources: YMF, CCZ, GS; Supervision: YMF, GS, JHQ; Validation: YMF, WL, TW, WBH; Visualization: WL, TW, WBH; Writing-Original Draft Preparation: WL, TW, WBH; Writing-Review and Editing: YMF, CCZ.

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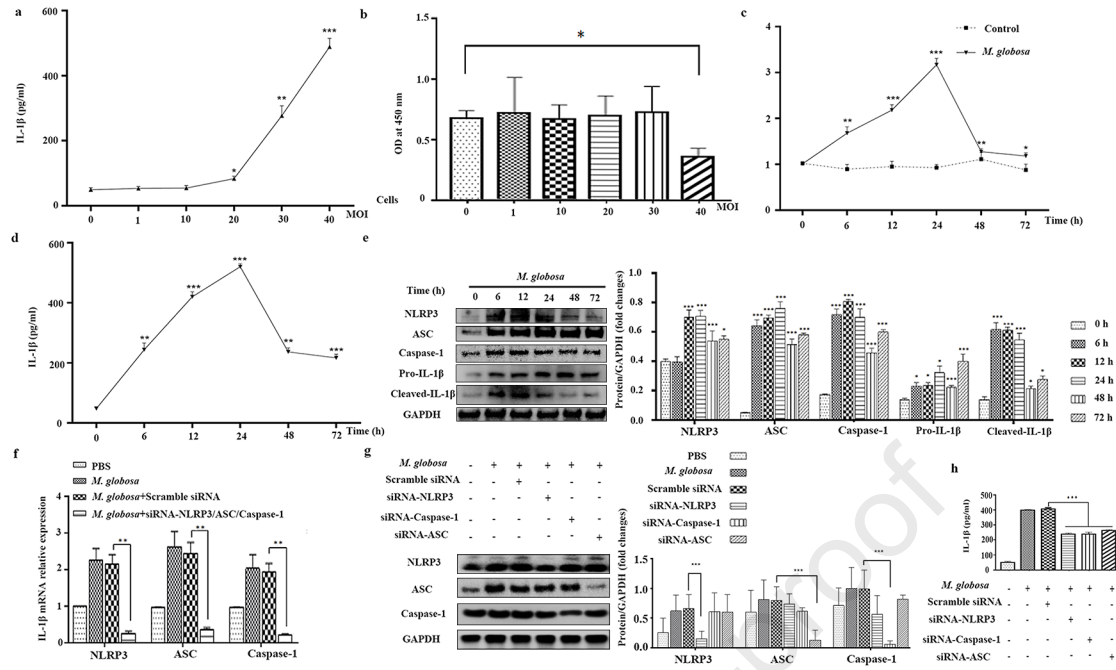
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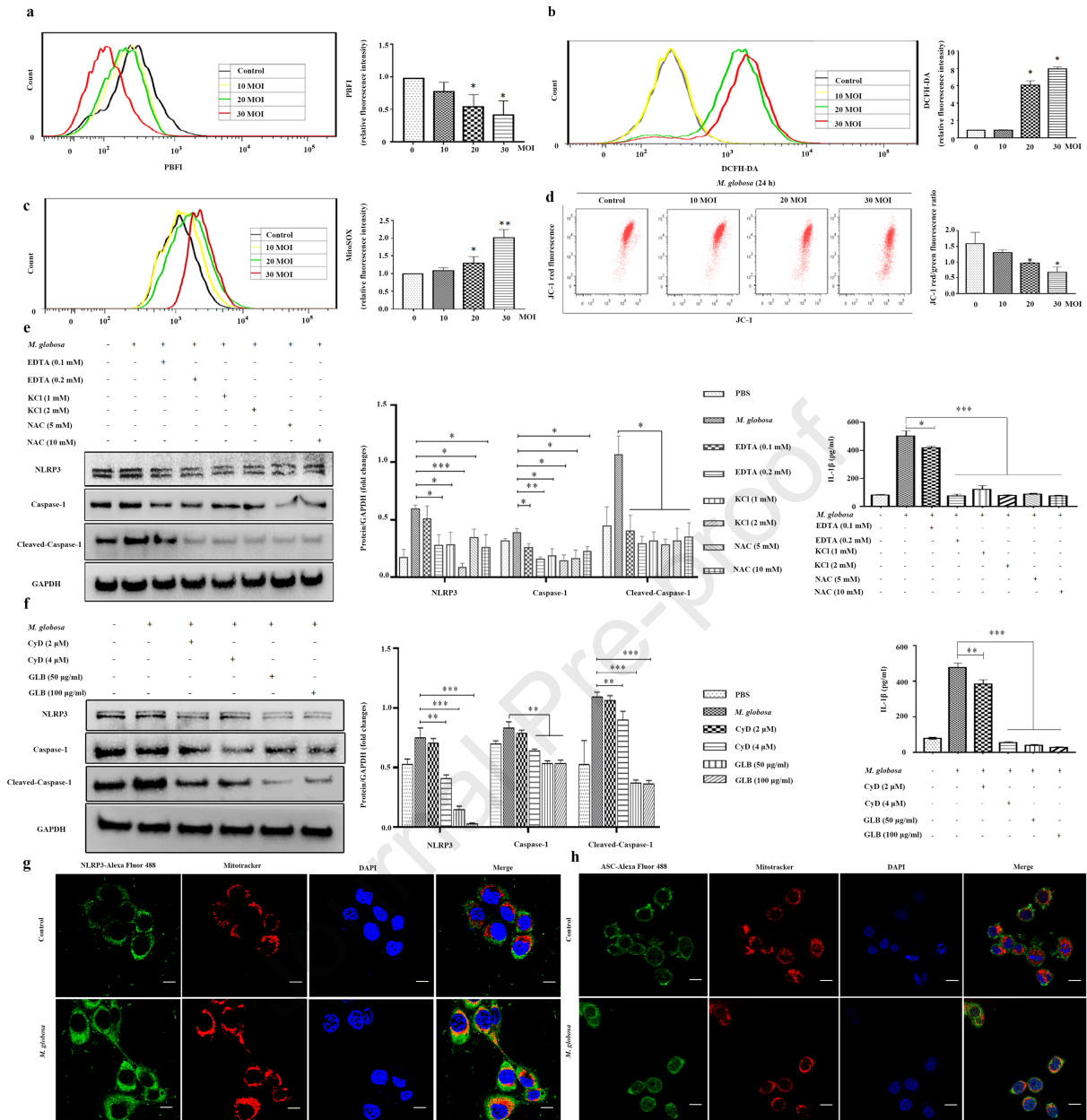
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### Figure legends

**Figure 1. MOI screening and NLRP3 inflammasome activation in *M. globosa*-infected SZ95 sebocytes.** (a, b) ELISA and CCK-8 showed IL-1 $\beta$  level (a) and cell viability (b) in 0-40 MOI *M. globosa*-infected SZ95 sebocytes for 24 h. (c, d) qRT-PCR (c) and ELISA (d) detected IL-1 $\beta$  level in 30 MOI *M. globosa*-infected SZ95 sebocytes for 0-72 h. (e) WB showed NLRP3 inflammasome and IL-1 $\beta$  expression in 30 MOI *M. globosa*-infected SZ95 cells. (f-h) qRT-PCR (f), WB (g) and ELISA (h) revealed NLRP3 inflammasome and IL-1 $\beta$  expression after NLRP3, ASC and caspase-1 silencing. Data are shown as mean  $\pm$  SD ( $n=3$ ). \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$ , compared to 0 h or scramble siRNA.

**Figure 2. Effect of secreted proteases, cytophagy, K<sup>+</sup> efflux, ROS generation, mitochondria damage, and NLRP3/ASC mitochondrial translocation on NLRP3 inflammasome activation of SZ95 sebocytes.** (a-d) Levels of K<sup>+</sup> (a), ROS (b), mitochondrial ROS (c), and MtMP (d) were determined by flow cytometry in SZ95 sebocytes infected with 10-30 MOI *M. globosa* for 24 h. (e, f) After pretreated with EDTA (0.1, 0.2 mM), KCl (1, 2 mM), NAC (5, 10 mM), cytochalasin D (CyD; 2, 4  $\mu$ M), and glibenclamide (GLB; 50, 100  $\mu$ g/ml) for 1 h, SZ95 cells were infected with 30 MOI *M. globosa* for 24 h. NLRP3, caspase-1, cleaved-caspase-1, and IL-1 $\beta$  were detected by WB and ELISA, respectively. (g, h) NLRP3 (g) and ASC (h) translocated into mitochondria 24 h after 30 MOI *M. globosa* infection. Bar=10  $\mu$ m. Data are presented as mean  $\pm$  SD. \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$ , compared to control or indicated group.





## SUPPLEMENTARY TEXT

### Supplementary Text 1 (Introduction)

The genus *Malassezia* are lipid-dependent basidiomycetous yeasts and currently comprise 18 species, with *M. restricta* and *M. globosa* being the most abundant on human skin (Vijaya Chandra et al., 2021). Apart from their commensal lifestyle, *Malassezia* can cause or aggravate several dermatoses, such as dandruff, seborrheic dermatitis, pityriasis versicolor, *Malassezia* folliculitis (MF), and atopic dermatitis (Sparber et al., 2020). Because of their main residence on the outer skin surface and follicular infundibulum, *Malassezia* are directly exposed to keratinocytes and various immunocytes including dendritic cells (DCs), macrophages, myeloid cells and  $\gamma\delta$ -T cells (Vijaya Chandra et al., 2021). Furthermore, *Malassezia*-host interactions are also mediated indirectly via secreted lipases, phospholipases and extracellular vesicles (Sparber et al., 2020). However, immunopathogenesis of *Malassezia*-associated disorders is not fully understood.

Pattern recognition receptors (PRRs) detect pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), and mainly consist of Toll-like receptors (TLRs), C-type lectin-like receptors (CLRs), NOD-like receptors (NLRs), and retinoic acid-inducible gene-I-like receptors (Patin et al., 2019).

Inflammasome is a cytosolic multiprotein complexes composed of a sensor, an adaptor ASC (apoptosis-associated speck-like protein containing a CARD), and an effector pro-caspase-1. NLRP1, NLRP3, NLRC4, absent-in-melanoma 2 (AIM2), and pyrin can serve as sensors to form canonical inflammasomes. Upon detection of

PAMPs or DAMPs, inflammasomes are assembled to activate caspase-1, trigger maturation and release of IL-1 $\beta$ , IL-18, gasdermin-D, and induce pyroptosis (Moretti and Blander, 2021).

TLRs and CLRs recognize carbohydrates in fungal cell wall (Sparber et al., 2020). Both CLR and MyD88 pathways are pivotal in regulation of adaptive immunity to *Malassezia* (Sparber et al., 2019). *Malassezia* allergen Mala s13 can bind to Dectin-1 and Dectin-2 on human monocyte-derived DCs (MoDCs) and macrophages. Dectin-1 could be involved in IL-1 $\beta$  signaling (Roesner et al., 2019). Dectin-2 and Mincle mediate *Malassezia* phagocytosis and cytokine production via binding cell wall constituents (Sparber et al., 2020). Different *Malassezia* species induce NLRP3 inflammasome activation and subsequent IL-1 $\beta$  secretion in human keratinocytes (Park et al., 2021), monocytes or MoDCs (Kistowska et al., 2014), and mouse bone marrow-derived macrophages (BMDMs) and DCs (BMDCs) (Wolf et al., 2021). Our previous study suggested a possible role of simultaneous activation of NLRP3, NLRC4 and AIM2 inflammasomes in MF pathogenesis (Liang et al., 2018). However, although their colonizing abundance might be related to sebaceous gland activity and lipid level on the skin (Vijaya Chandra et al., 2021), the effect of *Malassezia* on sebocytes has barely been investigated. The extracts of 5 *Malassezia* species upregulated IL-8 secretion in co-culture of human primary sebocytes and HaCaT keratinocytes (Kim et al., 2016). *Propionibacterium acnes* activated NLRP3 inflammasome in human SZ95 sebocytes (Li et al., 2014). Therefore, we postulated that *Malassezia* might also trigger NLRP3 inflammasome in human sebocytes.

**Supplementary Text 2 (Discussion)**

Sebocytes can serve as immunocompetent cells via expressing PRRs and producing cytokines, chemokines, antimicrobial peptides and lipids, and contribute to cutaneous inflammation (Picardo et al., 2015; Zouboulis et al., 2020). *P. acnes* induces IL-1 $\beta$  release via NLRP3 inflammasome activation in SZ95 and primary human sebocytes (Li et al., 2014). Just like human keratinocytes (Park et al., 2021), we found that 30 MOI *M. globosa* also evoke NLRP3 inflammasome-mediated IL-1 $\beta$  secretion in SZ95 sebocytes at 24 h. NLRP3 and IL-1 $\beta$  expression was remarkably increased in sebaceous glands of MF lesions. IL-1 $\beta$  level was decreased in *Nlrp3*<sup>-/-</sup> mice. These findings suggest that NLRP3 inflammasome-dependent IL-1 $\beta$  enhanced production caused by *M. globosa* in sebaceous glands might play a role in *Malassezia*-associated dermatoses.

Heat-inactivated *Malassezia* did not affect IL-1 $\beta$  production in human monocytes and MoDCs (Kistowska et al., 2014), while paraformaldehyde-fixed *M. globosa* failed to induce IL-1 $\beta$  secretion in mouse BMDCs (Wolf et al., 2021). Cytochalasin B prevented *Malassezia*-induced NLRP3 inflammasome activation in mouse BMDCs (Wolf et al., 2021). Heat/EDTA-treated *P. acnes* blocked IL-1 $\beta$  release in SZ95 sebocytes (Li et al., 2014). Similarly, preincubation with cytochalasin D or EDTA suppressed NLRP3 inflammasome activation and IL-1 $\beta$  release in this study, suggesting that yeast phagocytosis or *M. globosa*-derived proteases, at least metalloproteases, could be active in NLRP3 inflammasome activation of SZ95 sebocytes. *Malassezia*-secreted lipases, phospholipases and proteases may be important in pathogenicity and

environmental adaption (Li et al., 2020). Since sebocytes express proteinase activated receptor 2 (Li et al., 2014), the role of other *M. globosa*-derived proteases in NLRP3 inflammasome remains to be further studied.

KCl or glibenclamide prevented *Malassezia*-induced IL-1 $\beta$  production in human MoDCs (Kistowska et al., 2014) and mouse BMDCs (Wolf et al., 2021). However, glibenclamide did not affect *P. acnes*-induced IL-1 $\beta$  secretion in SZ95 sebocytes (Li et al., 2014). In this study, *M. globosa* exposure reduced K<sup>+</sup> and MtMP, but augmented ROS levels. Pretreatment with KCl, glibenclamide, or NAC restrained NLRP3 inflammasome activation and IL-1 $\beta$  release. Thus, NLRP3 inflammasome activation by *M. globosa* could depend on K<sup>+</sup> efflux and ROS production in SZ95 sebocytes. Furthermore, NLRP3 can relocate to mitochondria after stimulation, but it is currently unknown whether ASC colocalizes with NLRP3 in various subcellular sites (Moretti and Blander, 2021). Our observation revealed simultaneous mitochondrial translocation of NLRP3 and ASC in SZ95 sebocytes cocultured with *M. globosa* for 24 h, implying that their translocation might be inevitable for assembly and activation of NLRP3 inflammasome.

Fungal pathogens can engage multiple PRRs and their interactions to enhance or evade the immune response (Patin et al., 2019). Syk signaling is necessary for NLRP3 inflammasome activation by *Malassezia* in human and mouse DCs (Kistowska M, 2014; Wolf et al., 2021). *Malassezia* allergen Mala s13 stimulated IL-1 $\beta$  release through Dectin-1/Syk signaling in human MoDCs (Roesner et al., 2019). *Malassezia* activated NLRP3 inflammasome partially via Dectin-2, CARD9, but not Dectin-1, in LPS-

primed BMDCs from *Dectin-1*, *Dectin-2*, or *CARD9* deficient mice (Wolf et al., 2021). Dectin-1 rather than Mincle was needed for *Malassezia*-induced NLRP3 inflammasome activation in human MoDCs and murine BMDCs (Kistowska et al., 2014). Although Dectin-1, Dectin-2 and Mincle were unessential for driving type 17 immunity and fungal control of cutaneous *Malassezia* in knockout mice, *CARD9*<sup>-/-</sup> and *Myd88*<sup>-/-</sup> caused severe impairment of Th17 response to *Malassezia* (Sparber et al., 2019). TLR2/MyD88 pathway is involved in *M. furfur*-induced IL-8 and  $\beta$ -defensin 2 expression in human keratinocytes (Baroni et al., 2006) and *M. sympodialis*-activated mast cells (Selander et al., 2009). Cell-free extracts of *P. acnes* reinforced IL-8 secretion through TLR2-dependent signaling in SZ95 sebocytes (Huang et al. 2015). We found that TLR2, TLR4 and Dectin-1 immunoreactivity was increasable in sebaceous glands of MF lesions. Their expression was time-dependently enhanced in SZ95 sebocytes infected with 30 MOI *M. globosa*. TLR2/Dectin-1 inhibitors (C29/laminaran) and silencing inhibited NLRP3 inflammasome activation via downregulating TLR2/Dectin-1 expression in SZ95 cells. These results indicate that *M. globosa*-activated NLRP3 inflammasome could involve TLR2, TLR4, Dectin-1, Dectin-2 and their interactions in SZ95 sebocytes.

In conclusion, this study demonstrates that *M. globosa* can activate NLRP3 inflammasome and subsequent IL-1 $\beta$  release in SZ95 sebocytes via the first signal (TLR/MyD88 and Dectin/Syk signaling) and second signal (protease stimulation, phagosomal rupture, K<sup>+</sup> efflux, ROS production, mitochondrial damage, and NLRP3/ASC mitochondrial translocation) (**Supplementary Figure S5**). It is

necessary to further investigate the impact of *Malassezia*-induced NLRP3 inflammasome activation on pilosebaceous inflammation and potential target of NLRP3 inflammasome for treating *Malassezia*-associated dermatoses.

### **Supplementary Text 3 (Materials and Methods)**

#### **Agonists, inhibitors and antibodies**

Agonists, inhibitors and antibodies were listed in **Supplementary Table S2**.

#### **Immunohistochemistry**

Skin biopsies were obtained from 4 male MF patients and 4 healthy controls. Immunohistochemistry for TLR2, TLR4, Dectin-1, Dectin-2, NLRP3, and IL-1 $\beta$  was performed on paraffin-embedded sections as previously described (Liang et al., 2018). This research was approved by our hospital's institutional review board (No. PJ2015113KT), and the written informed consent was obtained from each participant.

#### **Cell culture**

SZ95 sebocytes (Zouboulis et al., 1999) were cultured in Sebomed medium (Biochrom, Berlin, Germany) supplemented with 10% fetal bovine serum (Gibco BRL, Rockville, MD, USA) and 5 ng/ml recombinant human epidermal growth factor (Invitrogen, Carlsbad, CA, USA) at 37°C in a 5% CO<sub>2</sub> incubator.

#### **Fungal culture**

*M. globosa* (CBS9597) was obtained from the Institute of Dermatology, Chinese Academy of Medical Sciences and Peking Union Medical College (Nanjing, China). The yeasts were cultured in modified Leeming-Notman broth medium (Sugita et al., 2005) for 7 days at 32°C, harvested by centrifugation at 3000 g for 3 min, washed thrice

in PBS, and diluted in sebocyte medium.

### **Transmission electron microscopy**

SZ95 sebocytes infected with 40 MOI *M. globosa* for 12 and 24 h were harvested, precipitated, fixed in glutaraldehyde and osmium tetroxide, stained with saturated uranyl acetate solution, dehydrated in serial ethanol, and embedded in Eponate 12 resin. Ultrathin sections were observed by JEM-1400 transmission electron microscopy (Jeol, Tokyo, Japan).

### **Cell viability and ELISA**

Cell viability and IL-1 $\beta$  levels were respectively detected by CCK-8 assay (Beyotime, Shanghai, China) and ELISA kit (Sangon, Shanghai, China).

### **qRT-PCR**

The infected SZ95 sebocytes were harvested at six timepoints (0 h, 6 h, 12 h, 24 h, 48 h, and 72 h). Total RNA was extracted from SZ95 sebocytes by TRIzol Reagent, and cDNA was synthesized by M-MLV Reverse Transcriptase (Invitrogen). Primer sets of 23 NLRs, TLR2, TLR4, Dectin-1, Dectin-2, AIM2, pyrin, ASC, caspase-1/4/5/8, Gasdermin D, IL-1 $\beta$ , IL-18, and hypoxanthine phosphoribosyltransferase 1 (HPRT1) (**Table S3**) were synthesized by Sangon. qRT-PCR was performed using SYBR Premix Ex Taq II kit (TaKaRa, Dalian, China) as previously described (Quan et al., 2018). Relative expression of target genes was normalized to reference gene HPRT1, and analyzed using  $2^{-\Delta\Delta C_t}$  method.

### **Western blot**

Total proteins were extracted from SZ95 sebocytes using RIPA lysis buffer and

determined using BCA protein assay kit (Beyotime). A total of 25 µg proteins were loaded on SDS-polyacrylamide gel and transferred onto PVDF membrane. The membrane was blocked with blocking solution (Sangon) and then incubated with primary antibodies TLR2, TLR4, Dectin-1, Dectin-2, NLRP3, ASC, caspase-1, cleaved caspase-1, IL-1 $\beta$ , and GAPDH at a 1:1000 dilution. Blots were incubated with HRP-conjugated secondary antibodies and visualized by enhanced chemiluminescence.

### **Immunofluorescence**

SZ95 sebocytes were seeded in confocal petri dishes, treated with 30 MOI *M. globosa* or other agents for 24 h, and then incubated with or without fluorescent probe 200 nM Mito-Tracker Red CMXRos (Beyotime) for 30 min. After washing twice with cold PBS, cells were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.2% Triton X-100 for 10 min, blocked with 5% BSA blocking buffer (Solarbio, Beijing, China) for 30 min, and incubated with following primary antibodies overnight at 4°C: TLR2 (1:150 dilution), TLR4 (1:200), Dectin-1 (1:200), Dectin-2 (1:200), NLRP3 (1:50) and ASC (1:250). Cells were incubated with Alexa Fluor 488-labeled secondary antibodies at room temperature for 1 h, mounted with DAPI-containing antifading mounting medium (Beyotime), and observed by confocal microscopy (Olympus, Japan).

### **Flow cytometry**

SZ95 sebocytes were seeded in 6-well plates and cultured with 10-30 MOI *M. globosa* for 24 h. K<sup>+</sup>, ROS, MtROS, and MtMP were respectively stained with fluorescent probes PBFI-AM (Invitrogen), DCFH-DA (Beyotime), MitoSOXTM Red (Invitrogen),

and JC-1 (Beyotime), and then determined by FACSCanto II™ flow cytometer (BD, CA, USA).

### **siRNA transfection**

TLR2, Dectin-1, NLRP3, ASC, caspase-1 targeted and scrambled siRNAs (**Table S4**) were synthesized by GenePharma (Shanghai, China). SZ95 sebocytes were transfected with siRNAs using Lipofectamine RNAi MAX (Invitrogen) according to manufacturer's protocol. Briefly, cells were seeded in 6-well plates and grown for 24 h. Diluted siRNA was added to diluted Lipofectamine RNAiMAX Reagent and incubated at room temperature for 5 min. The siRNA-lipid complex was added to cells, and incubated for 48 h at 37°C in 5% CO<sub>2</sub>. The knockdown efficiency was determined by qRT-PCR and WB.

### **Animals**

*Nlrp3*<sup>-/-</sup> and C57BL/6 WT mice were respectively purchased from Jackson Laboratory (Bar Harbor, ME, USA) and GemPharmatech (Nanjing, China). Animal experiments were approved by Animal Research Ethics of Guangdong Medical University (No. GDY2016065). *Nlrp3* deficiency was genetically confirmed by qRT-PCR with NLRP3 primer (forward: TGCCTGCTCTTTACTGAAGG; reverse: TTCCATTACAGTCACTCCAGATGT) designed by Jackson Laboratory. After intraperitoneal injection of 5% trichloroaldehyde (8 µl/g body weight), non-hemorrhagic dermabrasive wound (2×2 cm) on the shaved back was produced by gentle rubbing with fine sandpaper in 6-8-week-old mice of both genders. 1 ml of *M. globosa* inoculum (10<sup>8</sup> spores/ml) or PBS was applied topically to the abrasive wound once

daily for 7 consecutive days. Mice were sacrificed 3 d, 7 d, 14 d and 21 d postinfection.

Skin infection was verified by 10% KOH mount, fungal culture and periodic acid-Schiff stain. IL-1 $\beta$  expression was detected by qRT-PCR, WB and immunohistochemistry.

### **Statistical analysis**

All tests were repeated at least three times. Data were expressed as mean  $\pm$  standard deviation (SD) and analyzed by one-way ANOVA with LSD post-test or unpaired Student's *t* test using SPSS 23.0 software.

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### Supplemental Figure Legends

#### **Figure S1. Immunohistochemical and ultrastructural observation. (a)**

Overexpression of TLR2, TLR4, Dectin-1, NLRP3 and IL-1 $\beta$  in sebaceous glands of *Malassezia* folliculitis lesions compared with normal skin (diaminobenzidine stain). Bar=50  $\mu$ m. **(b)** SZ95 sebocytes cultured with 40 MOI *M. globosa* for 12 h and 24 h, transmission electron microscopy showed extracellular (green asterisks) and intracellular (red asterisks) spores, mitochondria (arrows) and lipid droplets (triangles). Bar=2  $\mu$ m.

#### **Figure S2. Role of TLR2, TLR4, Dectin-1, and Dectin-2 in NLRP3 inflammasome activation of SZ95 sebocytes infected with 30 MOI *M. globosa*. (a) TLR2, TLR4, Dectin-1 and Dectin-2 expression was determined by WB 0-72 h postinfection. (b-e)**

SZ95 sebocytes were pretreated with TLR2 agonist Pam3Cys-Ser-(Lys)4 (1  $\mu$ g/ml) and inhibitor C29 (25, 50  $\mu$ M) for 1 h **(b)** or TLR2 siRNA for 36 h **(c)**, as well as Dectin-1 agonist Zymosan (100  $\mu$ g/ml) and inhibitor Laminaran (5, 10  $\mu$ g/ml) for 1 h **(d)** or Dectin-1 siRNA for 36 h **(e)**, and then stimulated with 30 MOI *M. globosa* for 24 h. TLR2, Dectin-1, NLRP3, ASC, caspase-1, and IL-1 $\beta$  expression was detected by WB. Data are presented as mean  $\pm$  SD ( $n=3$ ). \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$ , compared to control group.

#### **Figure S3. Immunofluorescent staining of TLR2/4 and Dectin-1/2 in SZ95**

**sebocytes treated with *M. globosa* or agonists.** Immunofluorescence intensity of TLR2 **(a)**, TLR4 **(b)**, Dectin-1 **(c)**, and Dectin-2 **(d)** in SZ95 sebocytes infected with 30 MOI *M. globosa* or treated with TLR2 agonist Pam3Cys-Ser-(Lys)4 (1  $\mu$ g/ml), TLR4

agonist LPS (100 ng/ml), Dectin-1 agonist Zymosan (100 µg/ml), and Dectin-2 agonist Mannan (6 mg/ml) for 24 h. Bar=20 µm. Data are presented as mean ± SD ( $n=3$ ).

\* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$ , compared to control group.

**Figure S4. Impairment of *M. globosa*-induced skin inflammation in *Nlrp3*<sup>-/-</sup> mice.**

*Nlrp3*<sup>-/-</sup> and WT mice ( $n=3$ ) were topically inoculated with  $10^8$  spores/ml *M. globosa* or PBS on the abrasive wound of the shaved back once daily for consecutive 7 days.

(a-c) IL-1β expression was measured by qRT-PCR (a), WB (b) and immunohistochemistry (c) in *Nlrp3*<sup>-/-</sup> and WT mice 7 days postinfection. (d)

Cutaneous pathological changes in *Nlrp3*<sup>-/-</sup> and WT mice (hematoxylin-eosin stain).

Bar=50 µm. Data are presented as mean ± SD. \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$ , compared to PBS or WT group.

**Figure S5. Activation mechanism of *M. globosa*-induced NLRP3 inflammasome in SZ95 sebocytes.**

*M. globosa* drives the first signal to activate nuclear factor (NF)-κB pathway via TLR2/4-MyD88 and Dectin1/2-Syk signaling pathways, leading to upregulation of NLRP3 and pro-IL-1β transcription. The second signal-triggering mechanisms include protease stimulation, phagosomal rupture, K<sup>+</sup> efflux, ROS production, and mitochondrial damage, which promotes the assembly of NLRP3 inflammasome complex through recruiting ASC and pro-caspase-1. Thereafter, the activated NLRP3 inflammasome triggers proteolytic cleavage of pro-caspase-1 into cleaved caspase-1, which in turn cleaves pro-IL-1β and gasdermin-D into IL-1β and N-terminal fragment. The active N-gasdermin-D forms the cytomembrane pores, resulting in pyroptosis, IL-1β release and subsequent pilosebaceous inflammation.

**Table S1. qRT-PCR results of inflammasome-associated molecules in 30 MOI *M. globosa*-infected SZ95 cells during 72 h**

|             | Infection time |             |               |                |                  |                  |
|-------------|----------------|-------------|---------------|----------------|------------------|------------------|
|             | 0 h            | 6 h         | 12 h          | 24 h           | 48 h             | 72 h             |
| TLR2        | 1±0.04         | 1.32±0.1**  | 2.28±0.42***  | 3.73±0.34***   | 3.57±0.15*       | 8.35±0.3***abcd  |
| TLR4        | 1.02±0.21      | 1.49±0.26** | 1.22±0.24**   | 2.74±0.43***ab | 1.44±0.08**c     | 1.11±0.18*c      |
| Dectin-1    | 1.02±0.21      | 0.7±0.09**  | 3.88±0.41***a | 4.41±0.61***a  | 7.32±0.11***ab   | 11.4±0.69***abcd |
| Dectin-2    | 1.03±0.29      | 4.7±1.18*** | 4.08±0.21***  | 17.35±2.7**ab  | 12.65±1.69***abc | 4.64±1.42***c    |
| NAIP        | 1.01±0.02      | 0.75±0.16*  | 0.69±0.11*    | 0.8±0.06*      | 0.67±0.2*        | 0.68±0.17*       |
| NOD1        | 1±0.00         | 0.72±0.31*  | 0.65±0.25*    | 0.67±0.22*     | 0.65±0.17*       | 0.52±0.17*       |
| NOD2        | 1.01±0.01*     | 0.66±0.15*  | 0.58±0.13*    | 0.63±0.23*     | 0.73±0.14*       | 0.76±0.4*        |
| NLRC5       | 1.01±0.01      | 0.83±0.1*   | 0.68±0.13*    | 0.75±0.15*     | 0.62±0.22*       | 0.57±0.26*       |
| NLRP1       | 1.02±0.01      | 1.11±0.24   | 1.1±0.09      | 1.14±0.16      | 1.06±0.21        | 0.88±0.03*b      |
| NLRP2       | 1.04±0.02      | 1.05±0.05   | 1.01±0.06     | 0.56±0.02*ab   | 0.5±0.02*abc     | 0.52±0.05*ab     |
| NLRP3       | 1.01±0.01      | 2.1±0.321*  | 3.28±0.68*a   | 2.36±0.17*     | 1.78±0.35*b      | 1.43±0.18*abc    |
| NLRP14      | 1.03±0.02      | 0.52±0.26*  | 0.59±0.27*    | 0.57±0.27*     | 1.08±0.34        | 0.86±0.27        |
| NLRPX       | 1.02±0.00      | 1.1±0.16    | 0.99±0.31     | 0.53±0.08*a    | 0.55±0.19*a      | 0.54±0.2*a       |
| APAF-1      | 1.03±0.0196    | 0.95±0.06*  | 0.76±0.12*    | 0.64±0.08*a    | 0.65±0.13*a      | 0.57±0.04*a      |
| CIITA       | 1±0.00         | 0.78±0.11*  | 0.88±0.02*    | 0.76±0.13*     | 0.93±0.22        | 0.85±0.42        |
| ASC         | 1.01±0.01      | 1.62±0.23*  | 2.05±0.4*     | 2.7±0.47*a     | 3.03±0.07*ab     | 1.76±0.15*cd     |
| Caspase-1   | 1.01±0.01      | 1.71±0.14*  | 3.66±0.84*a   | 2.26±0.45*     | 1.78±0.05*b      | 1.69±0.04*b      |
| Caspase-4   | 1.01±0.01      | 1.08±0.19   | 1.07±0.1      | 1.63±0.23*ab   | 1.5±0.14*ab      | 1.52±0.23*b      |
| Caspase-8   | 1±0.00         | 0.5±0.03*   | 0.54±0.03*    | 0.6±0.22*      | 0.76±0.04*ab     | 0.38±0.10*abcd   |
| Gasdermin D | 1.01±0.0       | 1.49±0.26*a | 2.94±0.41*a   | 2.46±0.24*a    | 2.23±0.31*a      | 2.13±0.15*ab     |
| IL-1β       | 1.01±0.01      | 1.68±0.14*a | 2.18±0.12*a   | 3.17±0.14*ab   | 1.27±0.07*abc    | 1.18±0.07*abc    |
| IL-18       | 1±0.00         | 1.03±0.15   | 1.07±0.13     | 1.01±0.11      | 0.67±0.14*abc    | 0.7±0.01*abc     |

Data are shown as mean ± SD ( $n=3$ ). \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$  compared to 0 h; a-d,  $P<0.05$ , compared to 6 h, 12 h, 24 h, and 48 h, respectively.

**Table S2. Sources of agonists, inhibitors, and antibodies**

| Regents/antibodies  | Usage                                             | CAS no/<br>product code | Company                                     |
|---------------------|---------------------------------------------------|-------------------------|---------------------------------------------|
| Pam3Cys-Ser-(Lys)4  | TLR2 agonist                                      | ab142085                | Abcam, Cambridge, United Kingdom            |
| Lipopolysaccharide  | TLR4 agonist                                      | L2630                   | Sigma-Aldrich, St Louis, MI, USA            |
| Zymosan             | Dectin-1 agonist                                  | #12703                  | Cell Signaling Technology, Danvers, MA, USA |
| Mannan              | Dectin-2 agonist                                  | M7504                   | Invitrogen, Carlsbad, CA, USA               |
| C29                 | TLR2 inhibitor                                    | HY-100461               | MedChemExpress, Monmouth Junction, NJ, USA  |
| Laminaran           | Dectin-1 inhibitor                                | HY-119109               | MedChemExpress                              |
| EDTA                | Protease inhibitor                                | A100105                 | Sangon, Shanghai, China                     |
| Potassium chloride  | —                                                 | A100395                 | Sangon                                      |
| N-acetyl-L-cysteine | ROS inhibitor                                     | 616-91-1                | Sigma-Aldrich                               |
| Cytochalasin D      | Phagocytosis<br>inhibitor                         | 22144-77-0              | Sigma-Aldrich                               |
| Glibenclamide       | ATP-sensitive K <sup>+</sup><br>channel inhibitor | 10238-21-8              | Sigma-Aldrich                               |
| TLR2                | WB, IF, IHC                                       | MA5-32787               | Invitrogen                                  |
| TLR4                | WB, IF, IHC                                       | MA5-16216               | Invitrogen                                  |
| Dectin-1            | WB, IF, IHC                                       | PA5-34382               | Invitrogen                                  |
| Dectin-2            | WB, IF, IHC                                       | PA5-99873               | Invitrogen                                  |
| NLRP3               | WB                                                | #13158                  | Cell Signaling Technology                   |
| NLRP3               | IF, IHC                                           | ab4207                  | Abcam                                       |
| ASC                 | WB, IF                                            | ab155970                | Abcam                                       |
| Caspase-1           | WB, IF, IHC                                       | 22915-1-AP              | Proteintech Group, Rosemont, IL, USA        |
| Cleaved-Caspase-1   | WB                                                | #4199                   | Cell Signaling Technology                   |
| IL-1 $\beta$        | WB, IHC                                           | #12703                  | Cell Signaling Technology                   |
| IL-1 $\beta$        | ELISA, WB, IHC                                    | D220820                 | Sangon                                      |
| GADPH               | WB                                                | #2118                   | Cell Signaling Technology                   |

IHC, immunohistochemistry; IF, immunofluorescence; WB, Western blot

**Table S3. Human and mouse primer sequences for qRT-PCR**

| Gene                 | Forward primer (5'-3')  | Reverse primer (5'-3')  |
|----------------------|-------------------------|-------------------------|
| TLR2                 | TGATGCTGCCATTCTCATTC    | CGCAGCTCTCAGATTTACCC    |
| TLR4                 | TGAGCAGTCGTGCTGGTATC    | CAGGGCTTTTCTGAGTCGTC    |
| Dectin-1             | TGGCAACTGGGCTCTAATCT    | GCCATGGTACCTCAGTCTGG    |
| Dectin-2             | AAAAGAGGCTGGTTGTCCCT    | GCACCTTTGTCCCTTCACTG    |
| NAIP                 | AGTACTTTTTTCGACCACCCAGA | TAGTTGGCACCTGTGATTTGTC  |
| NOD1                 | ACTGAAAAGCAATCGGGAAC    | ACACACAATCTCCGCATCTTC   |
| NOD2                 | GCCTGATGTTGGTCAAGAAGA   | GATCCGTGAACCTGAACTTGA   |
| NLRC3                | GGAGCCTCACCAGCTTAGATT   | AGGCCACCTGGAGATAGAGAG   |
| NLRC4                | GGAAAGTGCAAGGCTCTGAC    | TGTCTGCTTCCTGATTGTGC    |
| NLRC5                | CACCCTGACCAACATCCTAGA   | TCTCTATCTGCCCACAGCCTA   |
| NLRP1                | ATACGAAGCCTTTGGGGACT    | ACAAAGCAGAGACCCGTGTT    |
| NLRP2                | CACCGAATGGATCTGTCTGA    | GTGGTCGTTCTTTCCGTGTT    |
| NLRP3                | AAAGGAAGTGGACTGCGAGA    | TTCAAACGACTCCCTGGAAC    |
| NLRP4                | CCAACGAGTTTGGCTGACTT    | GCTGTCGATGACGAACAAGA    |
| NLRP5                | CTGGGGAACGAAGGTGTAAG    | GCAAGTGCAAGAAAACCACA    |
| NLRP6                | CTGTTCTGAGCTACTGCGTGAG  | AGGCTCTTCTTCTTCTTCTCCTG |
| NLRP7                | TAACCCGTAGCACCTGTCATC   | GGTCTTCTTCCCAATGAAAGC   |
| NLRP8                | CGCTGGTGTGCTTTCTACTTC   | GGTCGGGTTTGGACATAATCT   |
| NLRP9                | CTAGCCTCTCCAGTCTGACAT   | GCGATGTCTTCACAACTTCAC   |
| NLRP10               | GTCACGGTGGAGGCTCTATTT   | CGAGAGTTGTCTTTCCAGTGC   |
| NLRP11               | GTGTTGCATGTGACGTTTCC    | TTTGTGCTCCCAATCTCC      |
| NLRP12               | CGACCTTTACCTGACCAACAA   | AGGTCCATCCCAAATAACCAG   |
| NLRP13               | ATGGTGTGTTGGACCGTATGT   | GCCAAATCTACCTCTGCTGT    |
| NLRP14               | CCGCTTGTACTTGTCTGAAGC   | GCCTCCATCTACTGGTGTGAA   |
| NLRPX                | TGGCCTTGTCTCAGCTCTTTA   | CACCAGTCCAGAACCATCTTG   |
| APAF-1               | CCACAGAGGCCAGTTGTTTT    | TATGGTGACCCATCCTGGTT    |
| CIITA                | ATAACTGCATCTGCGACGTG    | GCAGCCGTGAACTTGTGTGA    |
| AIM2                 | TAGCGCCTCACGTGTGTTAG    | GCTGAGTTTGAAGCGTGTG     |
| Pyrin                | ATGCGACCTAGAAGCCTTGA    | GATTCGCAGCTGTCTTTTCC    |
| ASC                  | CTCTGTACGGGAAGGTCCTG    | CAGGCTGGTGTGAAACTGAA    |
| Caspase-1            | GGGGTACAGCGTAGATGTGAA   | CTTCCCGAATACCATGAGACA   |
| Caspase-4            | TGTTCCCTATGGCAGAAGGC    | TGCCATGACCCGAACCTTGT    |
| Caspase-5            | TGTTAGCTATGGCTGAAGACAGT | TGTCCAGCCACGTTGTTCT     |
| Caspase-8            | ATGGAGACAAGGGCATCATC    | CCCCTGACAAGCCTGAATAA    |
| Gasdermin D          | TGCTGGGATGAAAAGAATCC    | ACTTGTGGGGATCAGAGACG    |
| IL-1 $\beta$         | CCACAGACCTTCCAGGAGAA    | GTGATCGTACAGGTGCATCG    |
| Mouse IL-1 $\beta$   | GTTCTTAAGGTGTTCCCTGGAGA | ATCCATTGCAGTGACTGTCTTA  |
| IL-18                | TGCATCAACTTTGTGGCAAT    | ATAGAGGCCGATTTCCCTTGG   |
| HPRT1                | GACCAGTCAACAGGGGACAT    | CTGCATTGTTTTGCCAGTGT    |
| Mouse $\beta$ -actin | TATGCTCTCCCTCACGCCATCC  | GTCACGCACGATTTCCCTCTCAG |

**Table S4. TLR2, Dectin-1, NLRP3, ASC, Caspase-1 targeted and scrambled siRNAs**

| siRNA          | Sense (5'-3')          | Antisense (5'-3')      |
|----------------|------------------------|------------------------|
| TLR2-386       | GCCUUGACCUGUCCAACAATT  | UUGUUGGACAGGUCAAGGCTT  |
| TLR2-2357      | GCAAGUAUGAACUGGACUUTT  | AAGUCCAGUUCAUACUUGCTT  |
| Dectin-1-219   | GAAGAUGGAUUAUCUCAAUTT  | AUUGAGUAUAUCCAUCUUCTT  |
| Dectin-1-752   | GCCCUCAUUAUAGUAUUUGUTT | ACAAAUACUAUAUGAGGGCTT  |
| NLRP3          | CAGGUUUGACUAUCUGUUCU   | AGAACAGAUAGUCAAAACC    |
| ASC            | CGGGAAGGUCCUGACGGAUGA  | UCAUCCGUCAGGACCUUCCCG  |
| Caspase-1      | CCAAUAAUGGACAAGUCAATT  | UUGACUUGUCCAUAUAUUGGTT |
| Scramble siRNA | UUCUCCGAACGUGUCACGUTT  | ACGUGACACGUUCGGAGAATT  |