JNK1 negatively controls antifungal innate immunity by suppressing CD23 expression

Xueqiang Zhao1,4, Yahui Guo1,4, Changying Jiang2, Qing Chang1, Shilei Zhang3, Tianming Luo1, Bin Zhang1, Xinming Jia3, Mien-Chie Hung2, Chen Dong1 & Xin Lin1,2

Opportunistic fungal infections are a leading cause of death among immune-compromised patients, and there is a pressing need to develop new antifungal therapeutic agents because of toxicity and resistance to the antifungal drugs currently in use. Although C-type lectin receptor– and Toll-like receptor–induced signaling pathways are key activators of host antifungal immunity, little is known about the mechanisms that negatively regulate host immune responses to a fungal infection. Here we found that JNK1 activation suppresses antifungal immunity in mice. We showed that JNK1-deficient mice had a significantly higher survival rate than wild-type control mice in response to Candida albicans infection, and the expression of JNK1 in hematopoietic innate immune cells was critical for this effect. JNK1 deficiency leads to significantly higher induction of CD23, a novel C-type lectin receptor, through NFATc1-mediated regulation of the CD23 gene promoter. Blocking either CD23 upregulation or CD23-dependent nitric oxide production eliminated the enhanced antifungal response found in JNK1-deficient mice. Notably, JNK inhibitors exerted potent antifungal therapeutic effects in both mouse and human cells infected with C. albicans, indicating that JNK1 may be a therapeutic target for treating fungal infection.

Every year, invasive fungal infections kill approximately one and a half million people worldwide1,2. C. albicans is the most frequent fungal species isolated from infected patients3. Increasing numbers of immunocompromised individuals, including individuals with HIV, recipients of organ transplants and patients with cancer treated with chemotherapy, together with limited numbers of antifungal drugs for use in clinical application and drug resistance are the main reasons for the high morbidity and mortality associated with disseminated candidiasis4,5. Therefore, understanding how the host immune system fights fungal infections is crucial for the development of novel immune-response-based therapies2,5.

Pattern recognition receptors—including Toll-like receptors (TLRs), C-type lectin receptors (CLRs), Nod-like receptors (NLRs) and RIG-I-like receptors (RLRs)—initiate the host immune response against invading pathogens6. Previous studies indicate that CLRs play critical roles in recognizing fungal surface components, leading to induction of the host antifungal immune responses5–8. CLRs, including dectin-1, dectin-2 and dectin-3 (also named MCL), recognize the various carbohydrate glycoprotein components of the fungal cell wall, such as β-glucan or α-mannan, which trigger downstream signaling cascades that are essential for inducing protective immunity against fungi in the host9–14. Activation of spleen tyrosine kinase (Syk) through CLRs triggers CARD9–BCL10–MALT1 (CBM)-complex-dependent NF-κB signaling in macrophages or dendritic cells (DCs), which then leads to the release of proinflammatory cytokines—including tumor necrosis factor (TNF)-α, IL-6 and IL-17, among others15,16. Phagocytosis, reactive oxygen species (ROS) production, neutrophil recruitment and inflammasome activation have been shown to play critical roles in the fungal killing process6–8. Recently, three groups, including ours, have reported that activated CLRs are rapidly targeted for lysosome-mediated degradation in response to fungal infection17–19.

c-Jun N-terminal kinases (JNKs) play important roles in T cell activation and T helper cell differentiation, cell apoptosis, obesity, insulin resistance and tumorigenesis20–23. Many efforts have identified various ATP-competitive or ATP-noncompetitive JNK inhibitors24,25. Although studies have shown that JNK can be activated by various pattern recognition receptors22, the functional roles of JNK in innate immune responses have not been well characterized. In particular, the role of JNK activation in host antifungal responses has not been studied. Here we report that JNK1 negatively regulates the host antifungal innate immune response through suppression of CD23 expression and may serve as a therapeutic target in fungal infection.

RESULTS

JNK1 negatively regulates host antifungal innate immune responses in vivo

To investigate the role of JNK in response to fungal infection, bone marrow–derived macrophages (BMDMs) were stimulated with the yeast or hyphal form of C. albicans and the fungal cell wall component zymosan or α-mannan. Each stimuli can effectively induce JNK1 and JNK2 (JNK1/2) phosphorylation (Fig. 1a). To further characterize the role of JNK function in the host immune response to fungal infection, we intravenously infected JNK1 knockout (KO), JNK2 KO and

1Institute for Immunology, Tsinghua University School of Medicine, Beijing, China. 2Departments of Molecular and Cellular Oncology, The University of Texas MD Anderson Cancer Center, Houston, Texas, USA. 3Department of Immunology, Tongji University School of Medicine, Shanghai, China. 4These authors contributed equally to this work. Correspondence should be addressed to X.L. (linxin307@tsinghua.edu.cn) or X.Z. (zhaoxueqiang@tsinghua.edu.cn).

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Figure 1  JNK1 negatively regulates the antifungal innate immune response. (a) WT BMDMs were stimulated with the yeast (multiplicity of infection (MOI) = 10) and hyphal (MOI = 0.1) forms of *C. albicans*, plate-coated zymosan (100 µg/ml) or α-mannan (100 µg/ml) for the indicated time. Cell lysates were analyzed by immunoblotting for the indicated proteins. The phosphorylated forms of proteins are preceded by “p”. (b,c) JNK1 KO (n = 8), JNK2 KO (n = 8) and control, WT (n = 8) mice were intravenously injected with 2 × 10⁵ CFU of *C. albicans* per mouse. (b) Mouse survival was monitored and is plotted. (c) Kidney fungal load was assayed 2 d after infection. Each dot represents data from a single mouse, n = 4 for each group in c. (d) Kidney sections from *C. albicans*-infected JNK1 KO and WT mice were stained with H&E, periodic acid–Schiff (PAS) or Ly-6G (neutrophil marker). Insets show regions of fungal inflammation, fungal growth and neutrophil infiltration, respectively, and are higher-magnification views of the boxed areas. Representative images of at least three replicates are shown. Scale bars, 500 µm (50 µm in insets). (e) The inflammatory score based on renal immune cell infiltration and tissue destruction, the fungal burden and the percentage of the area positive for Ly-6G are shown. n = 3 mice for each group, and three sections per kidney were analyzed. (f) BM cells from JNK1 KO (n = 10) and WT (n = 10) mice were intravenously injected into irradiated recipient mice separately. Seven weeks later, mice were intravenously infected with 2 × 10⁵ CFU of *C. albicans*. Survival of these mice was monitored. WT–KO mice are JNK1 KO irradiated mice that received WT BM cells. (g) JNK1 WT/Rag1 KO (n = 7) and JNK1 KO/Rag1 KO (n = 8) mice were intravenously infected with 2 × 10⁵ CFU of *C. albicans* per mouse. Survival of these mice was monitored. In b,f,g, the log-rank test was performed; in c, the two-tailed unpaired t-test was performed; in e, the multiple t-test was performed. n.s. (not significant), P > 0.05; *P < 0.05, **P < 0.01, ***P < 0.001. In c,e, data are shown as means ± s.e.m.
Representative data from three biological replicates are shown. For each biological replicate, BMDMs from one mouse were used. In peptide (10 µg/ml) was added to cells for 48 h. NO production in culture supernatants at the indicated time was measured by nitrite assay kit. (c,d) The binding affinity between α-mannan or curdlan and NIH-3T3 cells stably expressing CD23, dectin-1, dectin-3 or mock control vector was determined by atomic force microscopy (AFM) by using probe pretreated with α-mannan (20 mg/ml) or curdlan (10 mg/ml) (d). Each dot indicates the maximum force of one cell touched with the coated probe and n = 15 for each group. The representative results of two independent experiments are shown in c and d. (e) WT, JNK1 KO and JNK2 KO BMDMs were stimulated with heat-inactivated C. albicans (MOI = 10) for the indicated time. NO production in culture supernatants at the indicated time point was measured by nitrite assay kit. (f) P30A or control (pCtrl) peptide (10 µg/ml) was added to C. albicans, and cells were treated for 48 h. NO production in culture supernatants was measured by nitrite assay kit. Representative data from three biological replicates are shown. For each biological replicate, BMDMs from one mouse were used. In c–f, data are shown as means ± s.e.m.

Figure 2 JNK1 suppresses CD23 induction by C. albicans. (a) WT and JNK1 KO BMDMs were stimulated with yeast-form C. albicans (MOI = 10) for 24 h or medium as a control. Total RNA was extracted, and RNA–seq analysis was performed. Shown are heat maps for selected gene panels. Arrows highlight the unregulated CD23 (Fcer2a) and Nos2 genes. (b) JNK1 KO, JNK2 KO and control, WT BMDMs were stimulated with yeast-form C. albicans (MOI = 10) for 48 h. Cells were washed and collected for FACS staining. Shown is CD23 staining for gated CD11b+ cells. Experiments were performed in triplicate. (c,d) The binding affinity between α-mannan or curdlan and NIH-3T3 cells stably expressing CD23, dectin-1, dectin-3 or mock control vector was determined by atomic force microscopy (AFM) by using probe pretreated with α-mannan (20 mg/ml) or curdlan (10 mg/ml) (d). Each dot indicates the maximum force of one cell touched with the coated probe and n = 15 for each group. The representative results of two independent experiments are shown in c and d. (e) WT, JNK1 KO and JNK2 KO BMDMs were stimulated with heat-inactivated C. albicans (MOI = 10) for the indicated time. NO production in culture supernatants at the indicated time point was measured by nitrite assay kit. (f) P30A or control (pCtrl) peptide (10 µg/ml) was added to C. albicans, and cells were treated for 48 h. NO production in culture supernatants was measured by nitrite assay kit. Representative data from three biological replicates are shown. For each biological replicate, BMDMs from one mouse were used. In c–f, data are shown as means ± s.e.m.

wild-type (WT) control mice with a sub-lethal dose of C. albicans. We found that JNK1 KO mice were more resistant to fungal infection than JNK2 KO or WT mice (Fig. 1b). Consistently, the fungal load in the kidneys of JNK1 KO mice was significantly lower than that in WT mice (Fig. 1c and Supplementary Fig. 1a). JNK1 KO mice also exhibited reduced renal inflammation and reduced numbers of C. albicans colony forming units (CFU) in the kidney (Fig. 1d,e and Supplementary Fig. 1b,c). The above findings were confirmed using two different doses of fungus in JNK1 KO mice and their heterozygous littermates (Supplementary Fig. 1d,e). These data suggest that deficiency of JNK1, but not JNK2, in the host leads to a boost in host antifungal immunity.

Myeloid lineage cells, including macrophages and DCs, are key effector cells against fungi during the first few days of fungal infection1-26. JNK1 has been reported to be ubiquitously expressed in most of the cell types in the human body22. To investigate the cellular basis of the JNK1-related antifungal effect, we generated bone marrow (BM)–chimeric mice by reconstituting lethally irradiated WT mice with syngeneic JNK1 KO BM or JNK1 KO mice with WT BM. WT mice reconstituted with JNK1 KO hematopoietic cells showed a phenotype similar to that of mice with total JNK1 deficiency in response to C. albicans infection (Fig. 1f and Supplementary Fig. 1f, KO–WT versus KO–KO). Hematopoietic cells comprise both innate and adaptive immune cells. To determine the contribution of JNK1 that is expressed in the adaptive immune system to the fungal infection phenotype, we generated JNK1 and Rag1 double-KO mice by crossing JNK1 KO mice with Rag1 KO mice. Notably, the phenotype of mice with JNK1 deficiency in the absence of adaptive immune cells closely resembled that of mice with whole-body JNK1 KO in response to C. albicans infection (Fig. 1g). Together, these data indicate that JNK1 negatively regulates the antifungal innate immune response in vivo.
JNK1 deficiency induces elevated CD23 expression and nitric oxide production in vitro

Proinflammatory cytokines, such as IL-6 and TNF-α, are reported to be key factors required for the innate immune system to protect the host from fungi. However, we did not detect significant differences in the levels of these proinflammatory cytokines after fungal infection in JNK1 KO mice when compared with WT mice using multiplex cytokine array analysis (Supplementary Fig. 2a), which we confirmed in vitro using BMDMs stimulated with C. albicans (Supplementary Fig. 2b). Activation of p38 and ERK was also comparable in WT and JNK1 KO cells (Supplementary Fig. 2c). To identify gene(s) that might be responsible for resistance to fungal infection in JNK1 KO mice, we performed RNA–seq analysis on WT and JNK1 KO BMDMs after stimulation with yeast-form C. albicans (Supplementary Fig. 3a).

Notably, we found that a novel C-type lectin gene, Fcer2a, was significantly upregulated in stimulated JNK1 KO cells when compared with WT control cells (Fig. 2a).

Fcer2a encodes the protein CD23, which was identified as the low-affinity receptor for IgE and also functions as a CLR. It is located on chromosome 19p13.1 and forms a gene cluster with Cd209a, which encodes DC-SIGN. To validate the RNA–seq data, we analyzed the expression of a series of CLR genes by quantitative real-time PCR and found that Fcer2a was the only gene that was significantly induced in JNK1 KO BMDMs cells compared with WT cells (Supplementary Fig. 3b). We confirmed upregulation of CD23 protein on the cell surface upon C. albicans stimulation by flow cytometry analysis (Fig. 2b).

As CD23 is also a CLR, we examined whether it can directly recognize surface components of fungi by performing a cellular binding assay. Indeed, we found that CD23 could bind both the yeast and hyphal forms of C. albicans (Supplementary Fig. 4a). Since the C. albicans cell wall is mainly composed of α-mannan and β-glucan, we performed atomic force microscopy (AFM) and ligand-binding assays to determine whether CD23-expressing cells and/or CD23 protein can directly bind α-mannan and β-glucan. We found that both cell-surface-expressed CD23 and purified, soluble CD23 could effectively bind to α-mannan and β-glucan (or Curdlan) in a manner similar to that of the known CLR s lectin-1 and lectin-3 (Fig. 2c,d and Supplementary Fig. 4b).

It has been reported that infection of human monocyte-derived macrophages with Mycobacterium avium increases membrane expression of CD23 (ref. 29) and that CD23 induces nitric oxide synthase (NOS) activity in human monocytes. Furthermore, CD23 and nitric oxide (NO) are involved in the killing of Leishmania and M. avium by human macrophages. Notably, Fcer2a, which encodes the inducible NOS (iNOS), was highly differentially expressed in JNK1 KO BMDMs compared with WT cells (Supplementary Fig. 4c). We confirmed upregulation of CD23 and NO production in JNK1 KO mice when compared with WT control mice (Fig. 2f).

Figure 3 Elevated CD23 and iNOS expression in JNK1 KO mice is responsible for the enhanced antifungal immune response. (a,b) WT and JNK1 KO mice (n = 3 mice for each group) were intravenously infected with 5 × 10^5 CFU of C. albicans. 48 h later, kidneys were isolated and extracts were made for total RNA or protein. (a) Nos2 levels were measured by quantitative, real-time PCR. (b) CD23 was detected by immunoblotting of kidney protein lysates. (c) JNK1 KO mice (n = 3 mice for each group) were intravenously inoculated with 5 × 10^5 CFU of C. albicans per mouse. Infected mice were intraperitoneally administered 200 mg per kg body weight p30A or control peptide daily for a total of three times. Kidneys were dissected, and CFU assays were performed to detect fungal load. (d) WT (n = 10), JNK1 heterozygous (Het)/CD23 KO (n = 9), JNK1 KO/CD23 heterozygous (n = 8) and JNK1 KO/CD23 KO mice (n = 9) were infected with 2 × 10^5 CFU of C. albicans. Survival of these mice was monitored and is plotted. (e) JNK1 KO mice were intravenously injected with 2 × 10^5 CFU of C. albicans per mouse. Infected mice (n = 7 mice for each group) were intraperitoneally administered 30 mg per kg body weight per day SMT (iNOS-specific inhibitor), 10 mg per kg body weight per day spermidine (NOS1-specific inhibitor) or PBS (Ctr) daily. Survival of these mice was monitored. (f) L-NAME (100 mg per kg body weight) or PBS (Ctr) was administered intraperitoneally daily to JNK1 KO mice infected with 2 × 10^5 CFU of C. albicans (n = 8 mice for each group). Survival of the mice was monitored and is plotted. In a, the multiple t-test was performed; in c, the two-tailed unpaired t-test was performed; in d–f, the log-rank test was performed. **P < 0.01, ***P < 0.001. In a,c, data are shown as means ± s.e.m.
Figure 4  JNK1 negatively regulates CD23 expression through elevated dectin-1-dependent NFAT activation. 

(a–c) WT and JNK1 KO BMDMs were stimulated with heat-inactivated C. albicans (MOI = 10). (a) Cells were collected after 1 h of stimulation and assayed for immunofluorescence staining of NFATc1 (red) or p65 (green). Nuclei were counterstained with DAPI (blue). Magnification, 200×; scale bars, 6.6 µm. (b) Statistical analysis of the percentage of NFATc1+ and p65+ cells, performed using 12 images for each group collected from three independent experiments. (c) Nuclear extracts were collected from cells at the indicated time and analyzed by immunoblotting with antibodies to NFATc1, NFATc2, p65 and PCNA. (d,e) WT, dectin-1 KO and dectin-3 KO BMDMs were stimulated with α-mannan (10 µg/ml), zymosan (20 µg/ml) or heat-inactivated C. albicans (MOI = 10). Cells were collected after 1 h of stimulation and assayed for immunofluorescence staining for NFATc1 and p65. Statistical analysis of the percentage of NFATc1+ (d) and p65+ (e) cells is shown; ten images for each group were collected from two independent experiments for the analysis. (f) BMDMs from JNK1 KO mice were stimulated with heat-inactivated C. albicans (MOI = 10) for 24 h. TPCA-1 (NF-κB inhibitor; 1 µM) or 11R-VIVIT (NFAT inhibitor; 10 µM) was added to cells when stimulation began. After stimulation, total RNA was extracted and CD23 mRNA expression was measured by quantitative real-time PCR and normalized to that of the internal control, Gapdh. (g) ChIP assays with the indicated antibodies followed by real-time PCR analysis for the CD23 promoter. Results are shown as means ± s.e.m. after normalization to input. (h) HEK293T cells were cotransfected with a construct containing the indicated CD23 promoter together with Renilla luciferase (R-Luc). 24 h after transfection, cells were treated with phorbol 12-myristate 13-acetate (PMA; 200 ng/ml) and ionomycin (500 ng/ml) for another 6 h. The NFAT inhibitor 11R-VIVIT (10 µM) was added 20 min before stimulation with PMA and ionomycin. Luciferase assays were performed, and luciferase activity was normalized to R-Luc activity. In b,d,e, the multiple t-test was performed; in f–h, the two-tailed unpaired t-test was performed. n.s., P > 0.05; *P < 0.05, **P < 0.01, ***P < 0.001. In b–h, data are shown as means ± s.e.m.
levels of Nos2 and Fcer2a were significantly higher in JNK1 KO mice than in WT mice (Fig. 3a and Supplementary Fig. 6a). CD23 protein levels in the infected kidney were also higher in JNK1 KO mice (Fig. 3b), and p30A treatment could efficiently inhibit CD23 expression in mice (Supplementary Fig. 6b). To evaluate whether CD23 is responsible for the protection against fungal infection found in JNK1 KO mice, we used both mice treated with CD23 blocking peptide and CD23 KO mice. The fungal burden of p30A-treated JNK1 KO mice was much higher than that of mice treated with control peptide (Fig. 3c and Supplementary Fig. 6c), which is consistent with our in vitro data. JNK1 and CD23 double-KO mice were generated by crossing CD23 KO mice with JNK1 KO mice, and the mice generated were infected with C. albicans. In accordance with the data for the p30A blocking peptide, the increase in the survival rate of JNK1 single-KO mice in comparison to WT mice was diminished in JNK1 and CD23 double-KO mice (Fig. 3d and Supplementary Fig. 6d). Interestingly, JNK1 and CD23 double-KO mice died faster than WT and CD23 single-KO mice for unknown reasons.

As iNOS expression was elevated in response to fungal infection in JNK1 KO mice, we treated C. albicans–infected JNK1 KO mice with the Nos1-specific inhibitor spermidine and the iNOS (Nos2)-specific inhibitor SMT (Supplementary Fig. 6e). We found that SMT, but not spermidine, significantly reduced the survival rate of these mice as compared with control treatment (Fig. 3e). This iNOS-specific effect was confirmed using another inhibitor, l-NAME, which also reduced the survival rate of infected JNK1 KO mice (Fig. 3f). These data indicate that induction of CD23 expression and CD23-dependent elevation of NO levels are responsible for the enhanced antifungal immune response found in JNK1 KO mice.

Monocytes/macrophages, DCs and neutrophils are all reported to be important components of the host antifungal immune response. We examined which types of cells showed increased expression of CD23 and iNOS in C. albicans–infected mice and found that expression of Fcer2a and Nos2 was significantly induced in BMDCs and DCs, but not in neutrophils, from JNK1 KO mice after fungal infection (Supplementary Fig. 7a). Neutrophil number was lower in JNK1 KO mice after fungal infection (Supplementary Fig. 7b,c), which may have been caused by the lower fungal burden and reduced inflammatory response present 7 d after initial infection. Notably, peritoneal macrophages from CD23 KO mice expressed less Nos2 mRNA than corresponding cells from heterozygous littermates (Supplementary Fig. 7d). The fungal load was much higher in the kidneys of CD23 KO mice than it was in WT mice after C. albicans infection (Supplementary Fig. 7e). ROS production and phagocytosis are also important processes in antifungal responses, and NO can interact with ROS and form peroxynitrite to efficiently kill fungi. We found that JNK1-deficient BMDMs produced higher amounts of ROS than WT cells upon C. albicans infection (Supplementary Fig. 8a), but the ability of these cells to perform phagocytosis was comparable (Supplementary Fig. 8b,c).

**JNK1 negatively regulates CD23 expression through dectin-1-induced NFAT activation**

We next investigated how JNK1 deficiency results in upregulated CD23 expression following fungal infection. It has been shown that JNK1-deficient T cells exhibit elevated NFAT activation upon stimulation and NFAT binding to the CD23 promoter regulates expression of this gene. Interestingly, stimulation with C. albicans can also trigger NFAT activation in macrophages and DCs. To determine the molecular mechanism by which CD23 expression is upregulated...
following fungal infection, BMDMs were stimulated with \textit{C. albicans} and NFAT activation was examined. More NFATc1 translocated to the nucleus in JNK1 KO cells than in WT cells upon stimulation with \textit{C. albicans}, although nuclear translocation of the p65 NF-κB subunit was comparable (Fig. 4a–c). Notably, JNK phosphorylation and NFAT activation were dependent on dectin-1, as dectin-1 KO BMDMs showed significantly reduced NFAT activation and JNK phosphorylation upon stimulation with intact \textit{C. albicans} or the dectin-1 ligand, zymosan, whereas p65 translocation was not affected by dectin-1 loss upon stimulation with either α-mannan or zymosan.

![Figure 6](image)

**Figure 6** JNK inhibitor promotes the antifungal response in human cells. (a) THP-1 cells were stimulated with yeast-form \textit{C. albicans} (MOI = 10) and treated with SP600125 (40 µM) or DMSO as a control for 24 or 48 h. Total RNA was extracted. CD23 and iNOS gene expression was measured by quantitative real-time PCR. (b,c) Human PBMC-derived monocytes were stimulated with yeast-form \textit{C. albicans} (MOI = 10) and treated with SP600125 (40 µM) or DMSO for 24 or 48 h. (b) Total RNA was extracted. CD23 and iNOS gene expression was measured by quantitative real-time PCR. (c) Cell supernatants were collected at 48 h, and \textit{in vitro} killing assays were performed. For a–c, data are representative of at least two independent experiments. (d) Proposed model for the mechanism by which JNK1 regulates the antifungal immune response. After initial infection, \textit{C. albicans} is sensed by the CLR (dectin-1). In the absence of JNK1, NFATc1 activation is highly induced. NFATc1 binds to the CD23 promoter and induces CD23 expression. This results in production of a large quantity of NO, which kills \textit{C. albicans}. However, in WT cells, the basal level of CD23 is expressed following infection. In a–c, the multiple \textit{t}-test was performed. * \( P < 0.05 \), ** \( P < 0.01 \), *** \( P < 0.001 \). In a–c, data are shown as means ± s.e.m.
JNK24,25,44. To investigate whether JNK inhibitors can efficiently boost its potential for therapeutic intervention through direct inhibition, and several studies using SP600125 have demonstrated SP600125 is one of the most extensively used ATP-competitive JNK inhibitors, and treatment with 11R-VIVIT in comparison to control treatment (Fig. 4f). However, TPCA-1, which inhibits NF-kB activation through 1xK kinase (1KK), hardly influenced CD23 induction. Since two putative NFAT-binding sites were found in the Fcer2a promoter (at −1,368 and −714 bp with respect to the transcription start site), we performed ChIP assays and found high-level binding of NFATc1 to the CD23 gene promoter in JNK1 KO cells following stimulation with C. albicans (Fig. 4g). To determine whether the elevated nuclear translocation of NFATc1 dictates transcription of the Fcer2a gene, we cloned the genomic promoter (approximately 1,500 bp) of Fcer2a and performed luciferase reporter assays. We found luciferase reporter activity and found that CD23 activation could be substantially induced when both potential NFAT-binding sites were present; adding the NFAT inhibitor 11R-VIVIT completely diminished this activation (Fig. 4h, −1,500). Deletion of the NFAT site at −1,368 bp severely impaired CD23 promoter activation, but additional deletion of the NFAT site at −714 bp did not further reduce promoter activity (Fig. 4h, −750 and −480). These data indicate that NFATc1 regulates CD23 expression by directly binding to the site at −1,368 bp in the CD23 promoter.

JNK inhibitors promote antifungal immune responses in vivo and in vitro
SP600125 is one of the most extensively used ATP-competitive JNK inhibitors, and several studies using SP600125 have demonstrated its potential for therapeutic intervention through direct inhibition of JNK24,25,44. To investigate whether JNK inhibitors can efficiently boost the antifungal innate immune response, WT mice were infected with C. albicans and then treated with two different doses of SP600125. Administration of both doses of SP600125 increased the survival rate in comparison with control mice (Fig. 5a). Treatment with JNK inhibitor SP600125 also significantly reduced the fungal burden (Fig. 5b) and increased Nos2 and Fcer2a (CD23) expression in the kidneys of infected mice (Fig. 5c,d). Moreover, SP600125 specifically inhibited JNK activation and had no additional effect on phosphorylation of other MAP kinases (p38 and ERK) (Supplementary Fig. 10a,b). To confirm the antifungal effect of the JNK inhibitor in vitro, BMDMs were pretreated with SP600125 and then stimulated with C. albicans. Cell surface staining showed higher induction of CD23 expression upon JNK inhibition than with control treatment (Supplementary Fig. 10c). Consistently, SP600125-treated WT BMDMs produced more NO than control-treated cells (Supplementary Fig. 10d); these cells also killed fungi more efficiently than cells treated with the control, as shown by a reduced number of live fungal colonies formed within the same time period (Supplementary Fig. 10e).

To further confirm the antifungal effect of JNK inhibition in vivo, we used another JNK inhibitor, JNK-IN-8 (ref. 45), which specifically blocks JNK activation (Supplementary Fig. 10a,b), to treat mice infected with the clinical C. albicans strain sc5314. Strikingly, JNK-IN-8 exhibited a greater protective effect in fungal infection than SP600125 (Fig. 5e). These results demonstrate that JNK inhibitors can promote antifungal immune responses both in vivo and in vitro.

JNK1 is a potential therapeutic target in fungal infection
To investigate whether JNK inhibitors have a therapeutic benefit in fungal infection, we infected WT mice with C. albicans by intravenous injection and then injected these mice with JNK-IN-8 via the tail vein 24 h later. We found significantly higher fungal burden in the kidneys of solvent-treated mice than in those of mice treated with the JNK inhibitor (Fig. 5f). To further confirm the therapeutic effect of JNK inhibitors, we first intravenously infected WT mice with C. albicans and, beginning 24 h later, treated the mice intraperitoneally with SP600125 or solvent control daily for four continuous days and monitored the mortality of these mice. We found that most of the control mice died within 10 d of infection, but mice treated with the JNK inhibitor were significantly more resistant to infection (Fig. 5g).

To test the relevance of our results in mice to humans, we infected the human monocytic cell line THP-1 and human PBMC-derived monocytes with C. albicans. Both THP-1 and primary human cells treated with JNK inhibitor showed significant upregulation of CD23 and iNos at the mRNA level (Fig. 6a,b) and killed fungi more efficiently in vitro, as shown by fewer live fungal colonies formed within the same time period (Fig. 6c). Thus, we conclude that JNK1 deficiency leads to upregulation of the CLR CD23 through dectin-1-dependent NFAT hyperactivation and that this elevated level of CD23 induces cells to produce more NO through direct recognition of fungal cell wall components, thereby leading to efficient elimination of the fungal infection (Fig. 6d). Therefore, JNK inhibition enhances antifungal immunity and may serve as a potential therapeutic strategy to protect from lethal C. albicans sepsis in the clinic.

DISCUSSION
In this study, we have shown that JNK1 functions as a negative regulator in innate immune responses against fungal infection. We found that JNK1-deficient mice are more resistant to fungal infection than WT mice and that JNK1 deficiency in myeloid cells is vital for this phenotype. CD23, a novel CLR, is significantly induced in the absence of JNK1 following fungal infection and plays a critical role in antifungal innate immunity. The greater induction of CD23 in JNK1 KO cells is dependent on increased activation of NFATc1, which binds to the CD23 promoter and induces CD23 expression in macrophages. The upregulated CD23 in turn induces a much higher level of production for NO, which kills C. albicans. Notably, JNK inhibitors show a potent antifungal effect both in vitro and in vivo through CD23 upregulation and induction of NO. Together, these findings suggest that JNK1 negatively regulates the innate immune response against C. albicans infection and that inhibition of JNK1 activation can enhance innate immunity against fungal infection.

JNK is involved in many physiological and pathological processes. The mechanisms by which JNK regulates T cell immune responses and T helper cell differentiation have been well studied using mice deficient for JNK1 or JNK2 (ref. 20). The role of JNK in survival signaling, cell death, cancer development and diabetes is well established31,24,25,46. Despite JNK being activated by a wide range of biological stimuli, including growth factors, cellular or oxidative stress, inflammatory cytokines and pathogens, the functional role of JNK in innate immunity, especially in responses to pathogens, has not been characterized. We show that JNK1 functions as a negative regulator of the host response to infection by the fungal pathogen C. albicans. Notably, our findings suggest potential utility for JNK inhibitors as a novel antifungal therapeutic approach.
Monocytes, which include macrophages, DCs and neutrophils, have been reported to play important roles in antifungal immune responses. During fungal infection, macrophages have a marked impact on the inflammatory environment by influencing expression of iNOS, proinflammatory cytokines and chemokines. DCs can kill fungi directly or present fungal components to organ-draining lymph nodes to prime CD4 T cells. When they encounter infectious particles, neutrophils initiate an antimicrobial killing program that includes phagocytosis and production of various toxic agents, including ROS. Our studies find that CD23-dependent iNOS expression in JNK1 KO mice after fungal infection is significantly elevated in macrophages and DCs, but not in neutrophils. Interestingly, the number of neutrophils decreases in JNK1 KO mice upon infection, which may be owing to the lower fungal burden and reduced inflammatory responses in the host 7 d after infection. These data highlight the importance of NO produced by macrophages in antifungal immunity. However, the role of CD23 in host defense and its regulation of iNOS expression remain to be further investigated.

There is increasing evidence indicating that CLRs recognize microbial surface components and induce host antifungal innate immune responses. Several CLRs that sense both fungal and mycobacterial infections have recently been characterized. For example, dectin-1 recognizes C. albicans yeast cells by binding to surface α-glucans, whereas dectin-2 forms a heterodimer with dectin-3 to sense the hyphal form through α-mannan. However, the function of other CLR family members needs to be characterized. In our study, we report that CD23, a novel CLR that is well known for its function as a low-affinity receptor for IgE, plays an unexpected role in antifungal innate immune responses through recognition of the surface components of fungal pathogens. We have found that CD23 is an inducible CLR and can recognize the fungal surface components α-mannan and β-glucan, which regulate antifungal innate immunity. In accordance with the role of CD23 as a novel pattern recognition receptor involved in antifungal innate immune responses, we have found that modulation of CD23 by either peptide inhibitors or genetic deletion of the gene encoding CD23 has strong effects on antifungal innate immunity. Our data also indicate that CD23 regulates Nos2 expression, with this activity likely contributing to its role in antifungal innate immunity. Together, these results suggest that CD23 has other functions besides its well-known role as a low-affinity receptor for IgE.

In summary, our findings shed new light on the function of JNK1 as a negative regulator of the immune response during fungal infection and may have far-reaching and translationally relevant implications for developing novel approaches to fight fungal infection with JNK inhibitors.

METHODS
Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
X.Z. and Y.G. performed most of the experiments. C.J. and T.L. performed some of the mouse experiments. Q.C. and S.Z. performed some of the in vitro experiments. B.Z. analyzed the RNA-seq data and helped prepare the figures. X.J., M.-C.H. and C.D. provided key reagents and insightful discussion. X.L. and X.Z. conceived the project and wrote the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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1. Brown, G.D. et al. Hidden killers: human fungal infections. Sci. Transl. Med. 4, 156rv13 (2012).
2. Kim, J.Y. Human fungal pathogens: why should we learn? J. Microbiol. 54, 145–148 (2016).
3. Wöhrich, M., Deepe, G.S. Jr. & Klein, B. Adaptive immunity to fungi. Annu. Rev. Immunol. 34, 115–148 (2016).
4. Gow, N.A., van de Veerdonk, F.L., Brown, A.J. & Netea, M.G. Candida albicans morphogenesis and host defense: discriminating invasion from colonization. Nat. Rev. Microbiol. 10, 112–122 (2011).
5. Underhill, D.M. & Pearlman, A.R. Inducible CLR and can recognize the fungal surface components α-glucan recognition and control of fungal immunity. Immunity 39, 324–334 (2013).
6. Sahio, S. et al. Dectin-1 is required for host defense against Pneumocystis carinii but not against Candida albicans. Nat. Immunol. 8, 39–46 (2007).
7. Sahio, S. et al. Dectin-2 recognition of α-mannans and induction of Th1 cell differentiation is essential for host defense against Candida albicans. Immunity 32, 681–691 (2010).
8. Taylor, P.R. et al. Dectin-1 is required for β-glucan recognition and control of fungal infection. Nat. Immunol. 8, 31–38 (2007).
9. Dambuza, I.M. & Brown, G.D. C-type lectin receptors in immunity: recent developments. Curr. Opin. Immunol. 32, 21–27 (2015).
10. Gross, O. et al. Card9 controls a non-TLR signaling pathway for innate anti-fungal immunity. Nature 442, 651–656 (2006).
11. Zhu, L.L. et al. E3 ubiquitin ligase Cbl-b negatively regulates C-type lectin receptor–mediated antifungal innate immunity. J. Exp. Med. 213, 1555–1570 (2016).
12. Xiao, Y. et al. Targeting CBLB as a potential therapeutic approach for disseminated candidiasis. Nat. Med. 22, 906–914 (2016).
13. Wirnsberger, G. et al. Inhibition of CBLB protects from lethal Candida albicans sepsis. Nat. Med. 22, 915–923 (2016).
14. Dong, C., Davis, R.J. & Flavell, R.A. MAP kinases in the immune response. Annu. Rev. Immunol. 20, 55–72 (2002).
15. Wagner, E.F. & Nebreda, A.R. Signal integration by JNK and p38 MAPK pathways in cancer development. Nat. Rev. Cancer 9, 537–549 (2009).
16. Arthur, J.S. & Ley, S.C. Mitogen-activated protein kinases in innate immunity. Nat. Rev. Immunol. 13, 679–692 (2013).
17. Han, M.S. et al. JNK expression by macrophages promotes obesity-induced insulin resistance and inflammation. Science 339, 218–222 (2013).
18. Bogoyevitch, M.A., Ngioe, K.R., Zhao, T.T., Yeap, Y.Y. & Ng, D.C. c-Jun N-terminal kinase (JNK) signaling: recent advances and challenges. Biochem. Biophys. Acta 1804, 463–475 (2010).
19. Davies, C. & Tourneur, C. Exploring the function of the JNK (c-Jun N-terminal kinase) signaling pathway in physiological and pathological processes to design novel therapeutic strategies. Biochem. Soc. Trans. 40, 85–89 (2012).
20. Brown, G.D. Innate antifungal immunity: the key role of phagocytes. Annu. Rev. Immunol. 29, 1–21 (2011).
21. Fujisawa, H. et al. The absence of IgE antibody-mediated augmentation of immune responses in CD23-deficient mice. Proc. Natl. Acad. Sci. USA 91, 6835–6839 (1994).
22. Soilleux, E.J., Barten, R. & Trowsdale, J. DC-SIGN, a related gene, DC-SIGNR, and CD25 form a cluster on 19p13. J. Immunol. 165, 2937–2942 (2000).
23. Mossalayi, M.D. et al. CD23 mediates antimycobacterial activity of human macrophages. Infect. Immun. 77, 6537–6542 (2009).
24. Aubry, J.P. et al. The 25-kDa soluble CD23 activates type III constitutive nitric oxide-synthase activity via CD11b and CD11c expressed by human monocytes. J. Immunol. 159, 614–622 (1997).
25. Le Meur-Henchoz, S. et al. CD23 regulates monocyte activation through a novel interaction with the adhesion molecules CD11b/CD18 and CD11c/CD18. Immunity 3, 119–125 (1995).
32. Vouldoukis, I. et al. IgE mediates killing of intracellular Toxoplasma gondii by human macrophages through CD23-dependent, interleukin-10 sensitive pathway. *PLoS One* 6, e18289 (2011).

33. Vouldoukis, I. et al. The killing of Leishmania major by human macrophages is mediated by nitric oxide induced after ligation of the FcεRI/CD23 surface antigen. *Proc. Natl. Acad. Sci. USA* 92, 7804–7808 (1995).

34. Rambert, J. et al. Molecular blocking of CD23 supports its role in the pathogenesis of arthritis. *PLoS One* 4, e4834 (2009).

35. Wirnsberger, G. et al. Jagunal homolog 1 is a critical regulator of neutrophil function in fungal host defense. *Nat. Genet.* 46, 1028–1033 (2014).

36. Underhill, D.M., Rossnagle, E., Lowell, C.A. & Simmons, R.M. Dectin-1 activates Syk tyrosine kinase in a dynamic subset of macrophages for reactive oxygen production. *Blood* 106, 2543–2550 (2005).

37. Vonk, A.G., Wieland, C.W., Netea, M.G. & Kullberg, B.J. Phagocytosis and intracellular killing of Candida albicans blastoconidia by neutrophils and macrophages: a comparison of different microbiological test systems. *J. Microbiol. Methods* 49, 55–62 (2002).

38. Romero-Puertas, M.C. & Sandalio, L.M. Nitric oxide level is self-regulating and also regulates its ROS partners. *Front. Plant Sci.* 7, 316 (2016).

39. Dong, C. et al. Defective T cell differentiation in the absence of Jnk1. *Science* 282, 2092–2095 (1998).

40. Debnath, I., Roundy, K.M., Weis, J.J. & Weis, J.H. Defining in vivo transcription factor complexes of the murine CD21 and CD23 genes. *J. Immunol.* 178, 7139–7150 (2007).

41. Knittl, C. et al. The CD23b promoter is a target for NF-AT transcription factors in B-CLL cells. *Biochim. Biophys. Acta* 1588, 41–47 (2002).

42. Goodridge, H.S., Simmons, R.M. & Underhill, D.M. Dectin-1 stimulation by Candida albicans yeast or zymosan triggers NFAT activation in macrophages and dendritic cells. *J. Immunol.* 178, 3107–3115 (2007).

43. Zanoni, I. & Granucci, F. Regulation and dysregulation of innate immunity by NFAT signaling downstream of pattern recognition receptors (PRRs). *Eur. J. Immunol.* 42, 1924–1931 (2012).

44. Bennett, B.L. et al. SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. *Proc. Natl. Acad. Sci. USA* 98, 13681–13686 (2001).

45. Lalaoui, N. et al. Targeting p38 or MK2 enhances the anti-leukemic activity of Smac-mimetics. *Cancer Cell* 29, 145–158 (2016).

46. Weston, C.R. & Davis, R.J. The JNK signal transduction pathway. *Curr. Opin. Cell Biol.* 19, 142–149 (2007).

47. Ersland, K., Wührich, M. & Klein, B.S. Dynamic interplay among monocyte-derived, dermal, and resident lymph node dendritic cells during the generation of vaccine immunity to fungi. *Cell Host Microbe* 7, 474–487 (2010).

48. Borregaard, N. Neutrophils, from marrow to microbes. *Immunity* 33, 657–670 (2010).
ONLINE METHODS

Antibodies and reagents. Antibodies against phospho-p38 (4631), phospho-ERK (9101), phospho-JNK (9251), total p38 (9212) and total JNK (9252) were purchased from Cell Signaling Technology; antibodies against p65 (sc-8008), PCNA (proliferating cell nuclear antigen) (sc-56), NFATc1 (sc-7294), NFATc2 (sc-7296), CD23 (sc-271900), ERK (sc-154), IκBα (sc-371) and tubulin (sc-8035) were from Santa Cruz Biotechnology. Dectin-1 and -dectin-3 monoclonal antibodies were generated by using the extracellular domain of dectin-1 or dectin-3 as an immunogen, as previously described11. TP-CA-1 (T1452) and α-mannan (M3640) were obtained from Sigma. Zymosan (tlrl-zyn) was obtained from Invivogen. NFAT inhibitor 11R-VIVIT (13855), JNK inhibitor SP600125 (10010466) and NO inhibitor l-NAME (80210) were obtained by Beyotime Biotechnology. PerCP-cyanine5.5-conjugated anti-mouse CD11b antibody (45-0112-82) was purchased from eBioscience, FITC-conjugated anti-mouse F4/80 (123108) and PE-conjugated anti-mouse IgG (45-0112-82) was purchased from Invitrogen. FITC-conjugated goat anti-mouse IgG (Fcy) (HI303) and control peptide (pCtr; SFNYNYA) were synthesized by Peptide 20 and GL Biosch. See Supplementary Table 1 for antibodies used in this study.

Expression plasmids. The sequences for mouse CD23, dectin-1 and dectin-3 were amplified by PCR using cDNA from mouse BMDMs as a template. The PCR-amplified fragments were inserted into a lentiviral vector, pLVX-conjugated anti-mouse F4/80 (123108) and PE-conjugated anti-mouse CD23 (101608) antibodies were from BioLegend, APC-Cy7-conjugated anti-mouse CD11b (557657) antibody was from BD Pharmingen. FITC-conjugated goat anti-mouse IgG (H+L) secondary antibody was purchased from Invitrogen (62-6511). CD23 blocking peptide (p30A; FHENWPS) and control peptide (pCtr; SFNYNYA) were synthesized by Peptide 20 and GL Biosch. See Supplementary Table 1 for antibodies used in this study.

Mice. JNK1 KO mice, JNK2 KO mice and CD23 KO mice were purchased from the Jackson Laboratory and were bred on a C57BL/6 background. To obtain JNK1 KO/CD23 KO and JNK1 KO/Rag1 KO mice, JNK1 KO mice were paired with CD23 or Rag1 KO mice, and subsequent intercrossing of their offspring led to generation of the double-KO mice. Wild-type C57BL/6 mice were purchased from Jackson Laboratory and bred in the facility. All mice were housed in the specific-pathogen-free animal facilities at MD Anderson Cancer Center and Tsinghua University. In all experiments described here, male and female mice aged 8–12 weeks were used. All mouse experiments were performed in compliance with institutional guidelines and according to the protocol approved by the Institutional Animal Care and Use Committee of The University of Texas MD Anderson Cancer Center and Tsinghua University. No statistical method was used to predetermine sample size. The experiments were not randomized and were not performed with blinding.

Bone marrow–chimeric mice. Six-week-old recipient mice were lethally irradiated by X-ray (550 rad × 2), and 5 × 106 BM leukocytes from donors were intravenously transferred to the mice. Chimeras were used for further experiments 7–8 weeks after the initial reconstitution.

Murine systemic candidiasis model. For in vivo C. albicans infection, mice were injected via lateral tail veins with 200 µl of a suspension containing different doses of C. albicans (SC5314) in sterile PBS (Hyclone). Mouse survival was monitored following infection. Fungal load was assessed by plating a series of dilution plates of homogenized kidneys on YPD plates.

Bone marrow–derived macrophage preparation. Primary cultures of BMDMs from mice were prepared as previously described11. Briefly, BM cells were isolated from the femurs and tibias of mice. Erythrocytes were removed using a hypotonic solution. Cells were cultured for 7 d in DMEM containing 20% FBS, 55 µM β-mercaptoethanol, streptomycin (100 µg/ml), penicillin (100 U/ml) and 30% conditioned medium from L929 cells expressing macrophage colony-stimulating factor. After 6–7 d of culture, flow cytometry analysis indicated that the cell population contained above 97% CD11b+F4/80+ cells.

Isolation of neutrophils, monocytes, macrophages and dendritic cells. BM and splenic neutrophils, monocytes, macrophages and DCs were first purified with CD45 MAC beads (Miltenyi, 130-052-301) and then purified by FACS sorting of cells labeled with antibodies specific for CD11b (BD, 553796), CD11c (BD, 550261), Ly-6G (eBioscience, 48-5931-82), and Ly-6C (BD, 561237). For each staining, 1 µg of antibody was diluted in 400 µl of FACS staining buffer (PBS supplemented with 2% FBS and 2 mM EDTA). Purified neutrophils, monocytes, macrophages and DCs were cultured in RPMI medium.

Immunoblotting assays. BMDMs were stimulated and lysed in lysis buffer (150 mM NaCl, 50 mM HEPES pH 7.4, 1 mM EDTA, 1% Nonidet P-40, protease inhibitors). Nuclear extracts or total cell lysates were subjected to SDS–PAGE and then blotted.

Quantitative PCR. Total RNA was isolated using TRizol (Invitrogen) and reverse transcribed using SuperScript III (Invitrogen). qPCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems). The amounts of transcript were normalized to those for Gapdh. Melting curves were run to ensure amplification of a single product. The primers used are listed in Supplementary Table 2.

RNA sequencing. RNA–seq was performed by Novogene. Sequencing libraries were generated using the NEBNext Ultra Directional RNA Library Prep Kit for Illumina. Clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumina). Sequencing was carried out on an Illumina HiSeq platform, and paired-end reads were generated. The filtered reads were aligned to the most recent version of the mouse reference genome, mm10, using TopHat (version 2.1.0). After alignment, the BAM files for each individual alignment were used to analyze the differential expression of genes with Cufflinks (version 2.2.1). Heat maps were generated with the gplots package in R (version 3.2.3).

Chromatin immunoprecipitation assays. ChIP assays were performed with a ChIP kit (Active Motif, 53009) as previously described49. The antibodies used for ChIP were as follows: NF-κB p65 (sc-109) and NFATc1 (sc-7294) from Santa Cruz Biotechnology. The resulting DNA was analyzed by real-time PCR. The PCR primers used are listed in Supplementary Table 2.

Cytokine measurement. Cytokine panels in mouse serum were detected with the Milliplex Map kit (Millipore, MCYTMAG-70K). All assays were performed according to the manufacturer's protocols, and mean fluorescence intensities (MFI) were obtained using the Luminex 200 system and analyzed with Bio-Plex software (Bio-Rad). TNF-α, IL-6 and IL-1β levels in the supernatant of the cells were measured with Ready-SET-GO ELISA kits (eBioscience). All samples were measured in triplicate according to the manufacturer's protocol.

Luciferase reporter assays. Luciferase assays were performed as described previously49. Briefly, HEK293T cells were transfected with 200 ng of the luciferase (firefly) reporter plasmid pGL3 containing different lengths of the CD23 promoter together with 4 ng of EF1α-promoter–dependent Renilla luciferase reporter. Cells were from ATCC and tested mycoplasma free using the PlasmoTest Mycoplasma Detection Kit from Invivogen. The transfected cells were cultured in DMEM containing 10% FBS for about 24 h. Cells were treated with PMA (200 ng/ml) and ionomycin (300 ng/ml) for another 6 h. NFAT inhibitor 11R-VIVIT (10 µM) was added 20 min before stimulation with PMA and ionomycin. Resulting cells were collected and lysed. Firefly and Renilla luciferase activities were measured with the Dual-Luciferase Reporter System (Promega), and Renilla luciferase activity was used to normalize for transfection efficiency.

Immunofluorescence staining. Cells were fixed with 4% paraformaldehyde (PFA), permeabilized with 0.25% Triton X-100 and blocked with 10% goat serum. Antibodies to NFATc1 (Santa Cruz, sc-7294; 1:100 dilution) and p65...
Histopathology. For histopathology analyses, kidneys were fixed in a 4% PFA solution, processed according to standard procedures, embedded in paraffin and sectioned. 5-µm sections were stained with H&E, PAS, Gomori methamine silver (GMS) or antibody to Ly-6G (MDL, MD6477-020; 1:200 dilution). Stained sections were scanned using an Olympus microscope (IX73) and evaluated for severity of inflammation and intralesional fungal burden, as described previously19. Renal inflammation was scored on the basis of H&E and PAS staining (proportion of renal parenchyma and/or pelvis involved by tubulointerstitial nephritis and/or pyelonephritis) as not significant (score 0), less than 10% (score 1), mild-to-moderate presence in 10–25% of inflammatory foci (score 2), moderate-to-significant presence in 25–50% of inflammatory foci (score 3) or significant presence in more than 50% of inflammatory foci (score 4). ImageJ software was applied to evaluate the extent of infiltration by Ly-6G+ cells in affected kidneys.

Detection of reactive oxygen species production. The production of ROS was assayed as described with minor modifications36. Briefly, 2 × 10^5 BMDMs were washed twice with PBS and resuspended in DMEM containing 10 µM DCFH-DA. Cells were incubated at 37 °C for 30 min; after incubation, cells were washed five times with DMEM to remove nonspecific binding. Cells were then infected with heat-killed C. albicans at MOI = 10 for different periods of time. The relative amount of ROS generated was detected every 10 min by flow cytometry measuring the MFI in the FL1 channel.

Phagocytosis of C. albicans and in vitro fungal killing assays. The phagocytosis assay was performed as described with minor modifications37. Briefly, 5 × 10^5 FACs-sorted cells were dispensed into 24-well plate and incubated at 37 °C for 2 h to create a monolayer of phagocytes, followed by gentle washing with culture medium to remove non-adherent cells. Resulting cells were incubated with 5 × 10^5 CFU of C. albicans (MOI = 1) at 37 °C for 15 min, and the supernatant was collected and the monolayer was washed gently with DMEM to remove unengulfed microorganisms. The supernatant and well wash medium, containing the non-phagocytized C. albicans, were combined and plated in serial dilutions on YPD agar plates. Plates were incubated overnight at 30 °C. The percentage of phagocytized microorganisms was defined as (1 – (CFU of unengulfed C. albicans/CFU at the start of incubation)) × 100. For phagocytosis of C. albicans by BMDMs, GFP-labeled C. albicans yeast was used50. BMDMs were stained with APC-conjugated CD11b antibody (Biorad, 101211; 1:400 dilution) and were then co-cultured with UV-cross-linked GFP-labeled C. albicans (MOI = 5) for 1 h at 37 °C. Adherent fungal cells were stained with trypan blue, and the phagocytosis rate for gated CD11b+ cells was determined by flow cytometry for GFP. In vitro fungal killing assays were performed as described before with minor modifications34. Briefly, cells were allowed to interact for 30 min at 30 °C with live C. albicans at a ratio of 0.4 yeast per macrophage. Unbound particles were removed, and cells were returned to the incubator for 4 h to allow fungal killing. Control plates were kept at 4 °C to provide a measure of live fungi in the wells. After incubation, medium was removed and cells were lysed by incubation for 5 min at 25 °C with water at pH 11. Lysis buffer was neutralized with excess PBS, and the CFU was determined by plating on YPD agar plates incubated overnight at 30 °C.

Measurement of nitric oxide concentration. Cell culture supernatants were collected and the concentration of NO was measured using an NO assay kit (S0021) from Beyotime Biotechnology based on the Griess reaction. All samples were prepared according to the manufacturer’s protocol.

Ligand binding assays. The ligand binding assay was performed as previously described31. In brief, ELISA plates were coated with α-mannan or β-glucan (40 mg/ml) overnight and 100 µl of reconstituted recombinant protein was added to each well. Bound proteins were detected by incubation with respective mouse monoclonal antibodies followed by HRP-conjugated goat anti-mouse IgG secondary antibody (ESABIO, BE0102). Tetramethylbenzidine (TMB) substrate solution from ebioscience ELISA kits was used, and reactions were stopped by the addition of 2N H2SO4. OD492 was read on a SpectraMax Plus 384 Microplate Reader.

Cellular binding assays. The cellular binding assay was performed as described with minor modifications14. Briefly, RAW264.7 cells stably expressing FLAG-tagged CD23, dectin-1 or dectin-3 were co-cultured with the GFP-labeled yeast (MOI = 5) or hyphal (MOI = 0.1) form of C. albicans for 10 min, and cells were washed four times with medium to remove unbound yeast (for yeast-form binding) or raw cells (for hyphal-form binding). Cells were from ATCC and tested mycoplasma free using the PlasmoTest Mycoplasma Detection kit from InvivoGen. Cells were then fixed with 4% PFA, permeabilized with 0.25% Triton X-100 and blocked with 10% goat serum. Antibody to FLAG (Abmart, M20008; 1:100 dilution) was used as the primary antibody, and Cy3-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch, 715-166-150; 1:1,000 dilution) was used as the secondary antibody. DAPI (Beyotime Biotech; 1:2,000 dilution) was used as a counterstain to visualize cell nuclei. Fluorescence was detected using a Zeiss LSM780 confocal laser scanning microscope.

Atomic force microscopy. Experiments were performed as described before52 using the JPK Cellhesion unit with minor modifications (set point, 0.5 nN; pulling length, 50 µm; delay mode, constant force; contact time, 5s). Probes (Nanoworld, ARROW-TL-50) were precoated overnight with anti-mannan (20 mg/ml) or curdlan (10 mg/ml). NIH-3T3 cells stably expressing CD23, dectin-1, dectin-3 or control vector were assayed with the pretreated probes. Cells were from ATCC and tested mycoplasma free using the PlasmoTest Mycoplasma Detection kit from InvivoGen. The maximum force for each touch was normalized and analyzed using JPK image processing software.

Isolation of human monocye-derived monocytes. Human MDMs were generated as previously described with minor modifications38. In brief, PBMCs from healthy donors were isolated from heparinized blood on Lymphoprep (STEMcell, 07851), a Ficoll-sodium-based density gradient medium. Monocytes were then separated using Monocyte Isolation Kit II (Miltenyi Biotec, 130-091-153) and cultured in RPMI 1640 medium at 37 °C. The protocol was approved by the Ethics Committee of Tsinghua University.

Statistical analysis. All values in the paper are given as means ± s.e.m. unless stated otherwise. All in vitro experiments were replicated at least three independent times, and all in vivo experiments were replicated more than twice unless stated otherwise. Statistical significance was calculated by two-tailed unpaired t test, multiple t test or log-rank (Mantel–Cox) test using GraphPad Prism software. Statistical significance was set on the basis of P value. n.s., P > 0.05; *P < 0.05, **P < 0.01, ***P < 0.001.

Data availability. The RNA–seq data have been deposited in the Gene Expression Omnibus (GEO) under accession GSE83265.

49. Zhao, X.Q. et al. C-type lectin receptor dectin-3 mediates trehalose 6,6′-dimycolate (TDM)-induced Mincle expression through CARD9/Bcl10/MALT1-dependent nuclear factor-κB activation. J. Biol. Chem. 289, 30052–30062 (2014).
50. Jia, X.M. et al. CARD9 mediates dectin-1-induced ERK activation by linking Ras-GRF1 to H-Ras for antifungal immunity. J. Exp. Med. 211, 2307–2321 (2014).
51. Sato, K. et al. Dectin-2 is a pattern recognition receptor for fungi that couples with the Fc receptor γ chain to induce innate immune responses. J. Biol. Chem. 281, 38854–38866 (2006).
52. Flach, T.L. et al. Alum interaction with dendritic cell membrane lipids is essential for its adjuvanticity. Nat. Med. 17, 479–487 (2011).

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