# Bypassing Pathogen-Induced Inflammasome Activation for the Regulation of Interleukin-1β Production by the Fungal Pathogen *Candida albicans*

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***Background.*** Interleukin (IL)–1β has an important role in antifungal defense mechanisms. The inflammasome is thought to be required for caspase-1 activation and processing of the inactive precursor pro-IL-1β. The aim of the present study was to investigate the pathways of IL-1β production induced by *Candida albicans* in human monocytes.

***Methods.*** Human mononuclear cells were stimulated with *C. albicans* or mutant strains defective in mannosylation or chitin. Receptors were blocked with specific antagonists, and the IL-1β concentration was measured.

***Results.*** Human primary monocytes produce bioactive IL-1β when stimulated with *C. albicans.* The transcription of IL-1β was induced through mannose receptor (MR), Toll-like receptor (TLR) 2, and dectin-1 but not through TLR4 and TLR9. *N*-mannan–linked residues, chitin, and β-glucan from *C. albicans* are important for IL-1β stimulation. Surprisingly, processing and secretion of IL-1β in monocytes did not require pathogen-mediated inflammasome activation, because of the constitutive activation of caspase-1 and the capability of monocytes to release endogenous adenosine-5'-triphosphate.

***Conclusions.*** This study is the first dissection of the molecular mechanisms of IL-1β production by a fungal pathogen. Transcription through mannan/chitin/MR and β-glucan/dectin-1/TLR2 induces production of IL-1β by *C. albicans* in human monocytes, whereas processing of IL-1β is mediated by constitutively active caspase-1.

*Candida* species are among the most common nosocomial bloodstream pathogens in the United States and Europe [1, 2], and candidemia is associated with a high crude mortality rate of ~40% [3, 4]. The main host defense mechanisms in systemic candidiasis are phagocytosis and the killing of *Candida albicans* by neutrophilic granulocytes, monocytes, and macrophages [5, 6]. The proinflammatory cytokines tumor necrosis factor–α (TNF-α) and interleukin (IL)–1β are essential for anti-*Candida* host defense through neutrophil recruitment and phagocytosis [7], and interferon-γ (IFN-γ) has been shown to be important for nitric oxide (NO) production by macrophages [8]. IL-1α– and IL-1β–deficient mice have increased mortality rates, with endogenous IL-1β and IL-1α required for the induction of protective inflammatory responses in disseminated candidiasis [9].

In contrast to other proinflammatory cytokines, IL-1β and IL-18 lack a signal peptide [10]. After transcription and translation, pro–IL-1β is processed by the caspase-1 protease; secretion that is dependent on the interaction of adenosine-5'-triphosphate (ATP) with the P2X7 receptor then follows [11]. It has been recently shown that activation of caspase-1 in human monocytic leukemia (THP-1) cells and mouse macrophages requires a protein complex known as the "inflammasome" [12, 13]. Detection of specific pathogen-associated molecular patterns (PAMPs) or danger signals by the in-

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flammasome is achieved by proteins of the nucleotide-binding oligomerization domain–like receptor (NLR) family, such as cryopirin (NALP3) or IL-1–converting enzyme (ICE) protease-activating factor (Ipaf), leading to a conformational change in caspase-1 and caspase-1 activation [14]. Caspase-1 activity is essential for host defense against infection with *Francisella tularensis* [15], *Legionella pneumophila* [16], *Shigella* species [17], and *Pseudomonas aeruginosa* [18]. In addition to IL-1$\beta$ and IL-18, IFN-$\gamma$ is also indirectly dependent on caspase-1 activity, because of its induction by IL-18 [19].

No component of *C. albicans* has yet been reported to be able to interact with the NLR receptors that form the protein platform of the inflammasome. Therefore, the formal possibility exists that *C. albicans* differs significantly from other pathogens in the way that IL-1$\beta$ is induced. In the present study, we investigated how IL-1$\beta$ is induced, processed, secreted, and regulated by *C. albicans* in human monocytes, as well as the extent to which caspase-1 plays a role in this pathway. We concluded that the conventional pathway of caspase-1 activation by the inflammasome complex is bypassed during host-*Candida* interaction.

## METHODS

***Volunteers.*** Blood samples were collected from 14 healthy, nonsmoking volunteers who were free of obvious diseases. After written informed consent was obtained, venipuncture was performed to collect blood into 10-mL ethylenediaminetetraacetic acid (EDTA) syringes (Monoject).

***Mice.*** Ipaf-deficient, NALP3-deficient, and caspase-1–deficient mice on a C57BL/6 background were previously described elsewhere [20–22]. Age-matched C57BL/6 mice were used as control mice. Animal experiments were approved by the Institutional Animal Care and Use Committee at St. Jude Children's Research Hospital (Memphis, Tennessee).

***Reagents.*** The irreversible caspase-1 inhibitor Ac-Tyr-Val-Ala-Asp-2,6 dimethylbenzoyloxymethylketone (YVAD) was purchased from AlexisBiochemicals, reconstituted in 10 mmol/L dimethyl sulfoxide (DMSO), and subsequently diluted to the desired concentration in medium (RPMI 1640). The proteinase 3 inhibitor and lipopolysaccharide (LPS) (*Escherichia coli* serotype 055:B5) were purchased from Sigma. LPS was repurified as described elsewhere [23]. Mouse anti–human monoclonal anti–Toll-like receptor (TLR) 4 HTA125 antibody (aTLR4) was provided by Kensuke Miyake (Saga Medical School, Saga, Japan). Mouse anti–human monoclonal anti-TLR2 antibody (aTLR2) was provided by Douglas Golenbock (University of Massachusetts, Boston). Mouse anti–human monoclonal anti-CD14 WT14 antibody (aCD14) was a gift of Wil Tax (Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands). Anti–dectin-1 antibody (aDectin-1) was a gift of Gordon Brown (University of Capetown, Capetown, South Africa). Mouse control antibody was purchased from Sigma. Chitin was prepared according to protocols described elsewhere [24]. *C. albicans* mannan (mannan) was provided by David L. Williams (East Tennessee State University, Johnson City, Tennessee). TLR9 was inhibited with oligodeoxynucleotide TTAGGG: 5'-TTTAGGGTTAGGGTTAGGGTTAGGG-3' (aTLR9) (InvivoGen). Syk inhibitor (Syk-i) was purchased from Calbiochem. P2X7 receptor was inhibited with oxATP (Sigma). In experiments using pharmacological inhibitors, control cells were treated with an equivalent concentration of vehicle (0.01%–0.1% DMSO).

**C. albicans *strains and mutants.*** *C. albicans* ATCC MYA-3573 (UC 820) [25] was used, unless otherwise indicated. *Candida* organisms were grown overnight in Sabouraud broth at 37°C, and cells were then harvested by centrifugation, washed twice, and resuspended in culture medium (RPMI 1640; ICN Biomedicals) [26]. To generate pseudohyphae, *C. albicans* blastoconidia were grown at 37°C in culture medium, which was adjusted to a pH of 6.4 by use of hydrochloric acid. Pseudohyphae were killed for 1 h at 100°C and were resuspended in culture medium to a hyphal inoculum size that originated from $10^6$ microorganisms/mL blastoconidia (referred to as "$10^6$ microorganisms/mL pseudohyphae") [26]. The *C. albicans* CAI4 strain (i.e., the wild-type strain), *och1*$\Delta$ null mutant (defective in outer, branched *N*-linked glycans) [27], *mnt1*$\Delta$ *mnt2*$\Delta$ double null mutant (lacking the 4 terminal *O*-linked $\alpha$1,2-mannosyl residues) [28], *mnn4*$\Delta$ null mutant (lacking phosphomannan) [29], CAF2 wild-type strain, and *chs1*$\Delta$ null mutant (defective in chitin) were used and have been well described elsewhere [30].

***Isolation of peripheral blood mononuclear cells (PBMCs) and in vitro stimulations.*** Separation and stimulation of PBMCs were performed as described elsewhere [31]. Cells were adjusted to $5 \times 10^6$ cells/mL. They then were incubated at 37°C in round-bottom 96-well plates (volume, 100 $\mu$L/well) with either heat-killed *C. albicans* ($10^6$ microorganisms/mL) or culture medium, with or without caspase-1 inhibitor at different concentrations. In some experiments, PBMCs were preincubated for 1 h with antibodies (anti-TLR4, 20 $\mu$g/mL; anti-TLR2, 20 $\mu$g/mL; anti-CD14, 20 $\mu$g/mL; and anti–Dectin-1, 10 $\mu$g/mL) or inhibitors (Syk-i, 50 nmol/L; chitin, 20 $\mu$g/mL; and mannan, 200 $\mu$g/mL) before stimulation with *C. albicans.* After 24 h, supernatants were collected and stored at −20°C until assayed. To investigate the role of endogenous ATP in the secretion of IL-1$\beta$, stimulations with RPMI and *C. albicans* were performed with or without oxATP for 24 h. For ATP measurements, PBMCs and macrophages were stimulated with RPMI or *C. albicans.* After 24 h, the supernatants were collected and directly measured.

***Production of cytokines by mouse macrophages.*** Resident peritoneal mouse macrophages were obtained aseptically with ice-cold PBS. Cells were resuspended in RPMI in a round-bottom 96-well plate ($10^5$ cells/well). For cytokine production, mouse macrophages were stimulated with culture medium as a

negative control or with heat-killed blastoconidia (ATCC MYA-3573; $10^7$ microorganisms/mL).

***Cytokine assays.*** IL-1$\beta$ concentrations were measured using a commercial ELISA kit (DY201; R&D Systems; Pelikine-Compact [Sanguin]), according to the manufacturers instructions. The presence of TNF-$\alpha$ was determined by specific radioimmunoassay (detection limit, 20 pg/mL), as described elsewhere [32]. Murine IL-1$\alpha$, IL-1$\beta$, and IL-6 concentrations were determined using specific radioimmunoassay, as described elsewhere [32]. ATP concentrations in the supernatants were assessed using a firefly luciferase assay (ATP determination kit; Invitrogen).

***Western blot assay.*** PBMCs ($10^7$ cells/well in a total volume of 1 mL) were incubated for 2 h (to evaluate caspase-1 activation) and 24 h (to assess pro-IL-1$\beta$ processing) with either heat-killed *C. albicans* ($10^6$ microorganisms/mL) or culture medium. THP-1 cells were cultured in RPMI medium and 10% fetal calf serum and were used for Western blot analysis of caspase-1. Cells were lysed in 100 $\mu$L of lysis buffer and were centrifuged (at 10,000 *g* for 5 min), and the protein content was determined by use of a bicinchonic acid protein assay (Pierce). Equal amounts of protein were loaded on 12% SDS-PAGE and transferred onto nitrocellulose membranes. For supernatants, 30-$\mu$L aliquots were loaded. Membranes were blocked in Tris-buffered saline–Tween (TBS-T) containing 3% w/v skim milk. For measurement of (pro-)IL-1$\beta$, membranes were incubated with anti–(pro-)IL-1$\beta$ polyclonal antibody (1/1000 dilution) (Cell Signaling). For caspase-1 quantitation, membranes were incubated with specific caspase-1 p10 polyclonal antiserum (1/500 dilution) (Santa Cruz). $\beta$-actin was quantified as an internal control by use of specific anti–$\beta$-actin polyclonal antiserum (1/1000 dilution) (Santa Cruz). Blots were washed and incubated with peroxidase-conjugated goat anti–rabbit IgG (1/1000 dilution). After the blots were washed 3 times with TBS-T, they were developed with Hyper ECL (GE Healthcare), according to the manufacturer's instructions.

***Quantitative polymerase chain reaction (qPCR).*** PBMCs were stimulated as described above. After 4 h, the supernatant was removed, and the cells were resuspended in 200 $\mu$L of RNAzolB RNA isolation solvent (Campro Scientific) and stored at $-80$°C. mRNA was isolated according to the manufacturer's protocol. cDNA was synthesized from 1 $\mu$g of total RNA by use of SuperScript reverse transcriptase (Invitrogen). Relative mRNA levels were determined using the Bio-Rad i-Cycler and the SYBR Green method (Invitrogen) The following primers were used: IL-1$\beta$ forward primer 5'-TGGCCCAGGCAGTCAGA-3' and reverse primer 5'-GGTTTGCTACAACATGGGCTACA-3' and $\beta$2M forward primer 5'-ATGAGTATGCCTGCCGTGTG-3' and reverse primer 5'-CCAAATGCGGCATCTTCAAAC-3' (Biolegio). Values are expressed as fold increases in mRNA levels, relative to those in unstimulated cells.

***Statistical analyses.*** Differences were analyzed by the Wilcoxon rank test or the Mann-Whitney *U* test. $P \leq .05$ was considered to denote significance. All experiments were performed at least twice, and the data presented are the cumulative result of all experiments performed. Data are expressed as the median $\pm$ interquartile range (IQR).

## RESULTS

***Induction of IL-1$\beta$ production in PBMCs by* Candida albicans.** Heat-killed *C. albicans* yeast cells, heat-killed *C. albicans* pseudohyphae, and live *C. albicans* are all capable of inducing secretion of IL-1$\beta$ in the supernatant of PBMCs (figure 1*A*). Mature IL-1$\beta$, but not pro-IL-1$\beta$, was found to be present in the supernatant by use of Western blot analysis (figure 1*B*). TaqMan reverse-transcriptase PCR analysis showed that heat-killed *C. albicans* yeast cells or pseudohyphae induced expression of IL-1$\beta$ mRNA in human PBMCs 4 h after stimulation. In line with the findings for IL-1$\beta$ secretion, stimulation with heat-killed pseudohyphae was lower than stimulation with heat-killed yeast cells (figure 1*C*).

***Caspase-1–dependent IL-1$\beta$ production induced by* C. albicans.** IL-1$\beta$, but not TNF-$\alpha$, production induced by *C. albicans* was reduced in the presence of the caspase-1 inhibitor (YVAD), in a dose-dependent manner (figure 2*A*). LPS-induced IL-1$\beta$ production was also inhibited by YVAD, with a reduction of 50%–60% observed (figure 2*A*). Interestingly, the proteinase 3 inhibitor alone did not result in a reduction in amounts of IL-1$\beta$ production; however, when used in combination with caspase-1 inhibitor, there was a slight yet statistically significant inhibitory effect on IL-1$\beta$ production (figure 2*A*). Neither the PR3 inhibitor nor the caspase-1 inhibitor alone induced IL-1$\beta$ production (data not shown). Western blot analysis confirmed that the lower IL-1$\beta$ level denoted a reduction in mature IL-1$\beta$ (figure 2*B*). Endogenous IL-1 can induce its own transcription [33]. Indeed, transcription of IL-1$\beta$ mRNA was reduced by the caspase-1 inhibitor (figure 2*C*).

***Constitutively active caspase-1 in human monocytes and endogenous ATP–dependent secretion of IL-1$\beta$.*** The possibility that *C. albicans* stimulation activates caspase-1 was investigated by Western blot analysis. Surprisingly, unstimulated PBMCs were found to have activated/processed caspase-1 (p35), and this was not up-regulated by heat-killed *C. albicans* yeast cells (figure 3*A*). To rule out the possibility that activation of caspase-1 occurred during the period of preparation of PBMCs, control experiments were performed using freshly isolated unstimulated PBMCs that were immediately lysed. Once again, caspase-1 was found to be present in its processed/active form (figure 3*B*). The THP-1 cells commonly used for studies of inflammasome activation showed no constitutively active caspase-1 (data not shown).

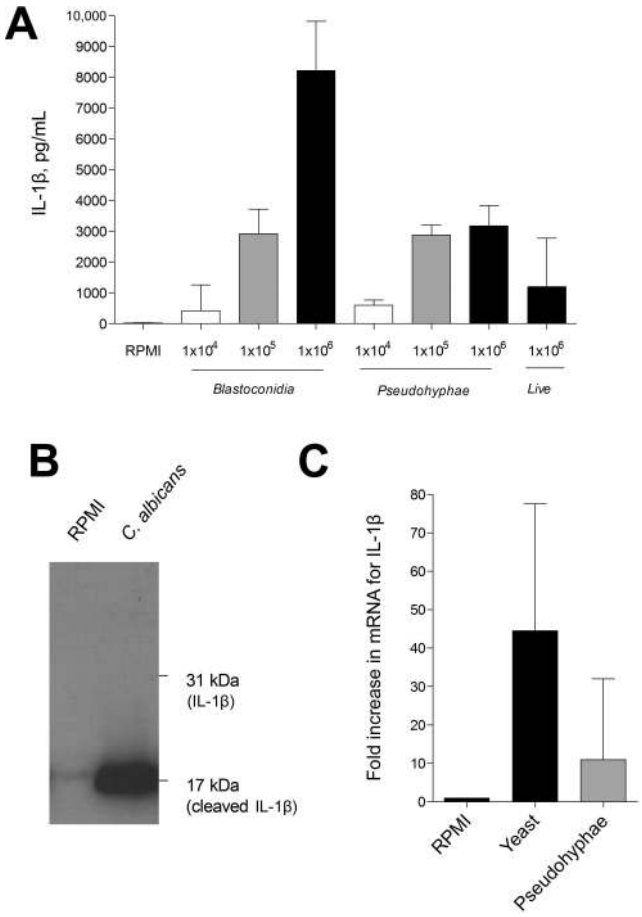


**Figure 1.** *Candida albicans*–induced production of interleukin (IL)–1$\beta$ in peripheral blood mononuclear cells (PBMCs). *A,* Human PBMCs were stimulated with heat-killed *C. albicans* yeast cells, heat-killed *C. albicans* pseudohyphae, or live *C. albicans* yeast cells (expressed as the no. of microorganisms per milliliter). ELISA was used to measure the production of IL-1$\beta$ in the supernatants of PBMCs incubated at 37°C for 24 h. *B,* Western blot analysis for IL-1$\beta$ in the supernatants of PBMCs stimulated with either control RPMI 1640 medium or heat-killed *C. albicans* yeast cells ($10^6$ microorganisms/mL). *C,* Reverse-transcriptase quantitative polymerase chain reaction analysis of mRNA for IL-1$\beta$ production in PBMCs stimulated for 4 h with heat-killed *C. albicans* yeast cells or pseudohyphae ($10^6$ microorganisms/mL) or with RPMI 1640 medium. In panel *A,* the data are pooled from 3 separate experiments involving a total of 8 healthy volunteers (median ± interquartile range; $^*P < .05$, by Wilcoxon rank test). Panel *B* presents findings from 2 separate experiments ($n = 4$), and panel *C* presents the average value of data from 2 separate experiments ($n = 4$).

We investigated the role of ATP in IL-1$\beta$ secretion by stimulating monocytes with heat-killed *C. albicans* in the presence or absence of a P2X7 receptor inhibitor (oxATP). oxATP strongly reduced IL-1$\beta$ stimulation by *C. albicans,* whereas the intracellular concentration was elevated (figure 3*C*). This finding suggested that endogenous ATP was present in the supernatants; indeed, endogenous ATP was released in the supernatants by monocytes but not by macrophages (data not shown). To assess whether induction of IL-1$\beta$ by *C. albicans* was dependent on the inflammasome, peritoneal macrophages from mice deficient in NALP3, Ipaf, or caspase-1 were stimulated with heat-killed *C. albicans.* The production of IL-1$\beta$ was dependent on caspase-1 (figure 3*D*). However, the absence of NALP3 or Ipaf did not alter IL-1$\beta$ production (figure 3*D*). In contrast to IL-1$\beta$, the production of IL-1$\alpha$ and IL-6 was not reduced in macrophages from mice deficient in either caspase-1 or NALP3/Ipaf (data not shown).

***Mediation of IL-1β production through TLR2/dectin-1, mannose receptor (MR), and chitin receptor pathways.*** The *C. albicans och1*Δ null mutant is defective in outer, branched *N*-linked glycan formations; the *mnt1*Δ *mnt2*Δ double null mutant lacks the 4 terminal *O*-linked $\alpha$1,2-mannosyl residues but has normal *N*-mannan; the *mnn4*Δ null mutant lacks phosphomannan; and the *chs1*Δ null mutant has a 70% reduction in the chitin content of the wall. PBMCs stimulated with the *och1*Δ null mutant or the *chs1*Δ null mutant released significantly lower amounts of IL-1$\beta$ than did the wild-type strains (figure 4*A*). In contrast, were no differences in IL-1$\beta$ production between the *mnt1*Δ *mnt2*Δ double null mutant and *mnn4*Δ null mutants. The *och1*Δ and *chs1*Δ null mutants expressed lower levels of mRNA for IL-1$\beta$ than did their wild-type strains (figure 4*B*), whereas transcription of the IL-1$\beta$ gene was not affected when PBMCs were stimulated with the *mnt1*Δ *mnt2*Δ and *mnn4*Δ mutants (data not shown).

To investigate which receptors were involved in IL-1$\beta$ production, PBMCs were stimulated with heat-killed *C. albicans* yeast cells in the presence of anti-TLR4 antibodies (aTLR4), aCD14, aTLR9, aTLR2, aDectin-1, and mannan. Chitin was used to block chitin-binding sites. Inhibition of the mannan and chitin-signaling pathway resulted in reduced IL-1$\beta$ production, whereas TLR4, CD14, and TLR9 inhibition did not modify IL-1$\beta$ production (figure 4*C*). A second mechanism of IL-1$\beta$ stimulation was represented by the TLR2/dectin-1 pathway, because blocking of TLR2 or dectin-1 inhibited the production of IL-1$\beta$. In addition, pharmacologic Syk inhibition also resulted in reduced IL-1$\beta$ production (figure 4*C*). *C. albicans* hyphae shield their $\beta$-glucans from recognition [34]. PBMCs stimulated with heat-killed *Candida* pseudohyphae showed no reduction in IL-1$\beta$ production when the dectin-1 receptor was blocked (figure 4*D*).

## DISCUSSION

In the present study, we demonstrated that *C. albicans*–induced production of IL-1$\beta$ in human PBMCs is dependent on the recognition of *N*-mannan–linked residues, chitin, and $\beta$-glucan components of the *C. albicans* cell wall. This recognition is mediated through the MR and dectin-1/TLR2. Caspase-1 is the main enzyme responsible for IL-1$\beta$ processing, whereas the serine protease PR3 plays a secondary role. However, although IL-1$\beta$ production is caspase-1 dependent, the regulation of its

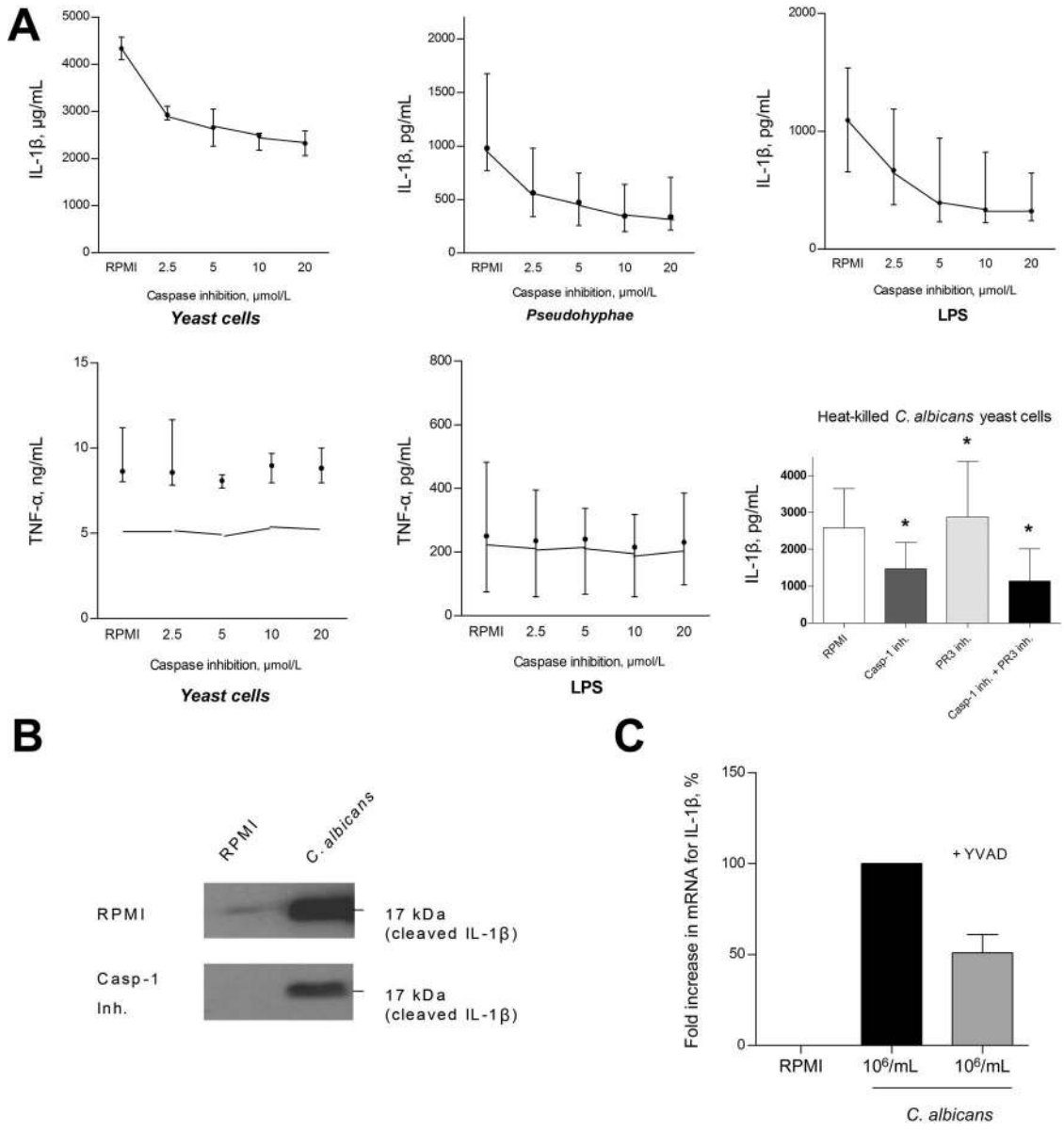


**Figure 2.** Caspase-1–dependent *Candida albicans*–induced interleukin (IL)–1$\beta$ production. *A,* Human peripheral blood mononuclear cells (PBMCs) were stimulated for 24 h with heat-killed *C. albicans* yeast cells or pseudohyphae ($10^6$ microorganisms/mL) or with lipopolysaccharide (LPS; 1 ng/mL), with or without various concentrations of the caspase-1 inhibitor (casp-1 inh.; YVAD) and with or without a PR3 inhibitor (PR3 inh.) or a combination of PR3 inh. and YVAD. Production of IL-1$\beta$ and tumor necrosis factor (TNF)–$\alpha$ in the supernatants was measured by ELISA after incubation at 37°C for 24 h. *B,* Western blot analysis was performed for the detection of IL-1$\beta$ in supernatants from PBMCs stimulated for 24 h with heat-killed *C. albicans* yeast cells ($10^6$ microorganisms/mL) with or without a casp-1 inhibitor (20 $\mu$mol/L). *C,* Reverse-transcriptase quantitative polymerase chain reaction analysis of mRNA for IL-1$\beta$ in PBMCs stimulated for 4 h with *C. albicans* yeast cells ($1 \times 10^6$ microorganisms/mL) with or without a caspase-1 inhibitor. In panel *A,* data are pooled from 3 separate experiments involving a total of 7 healthy volunteers (median $\pm$ interquartile range; $^*P < .05$, by Wilcoxon rank test). The data in panels *B* and *C* are the average values from 2 separate experiments ($n = 4$).

production mainly takes place at the level of transcription. This is because of the constitutive activation of caspase-1 in human PBMCs, as well as the capability of PBMCs to release endogenous ATP, leading to the secretion of the processed IL-1$\beta$. The implication of these findings is that *Candida*-induced activation of the inflammasome complex is not required for the induction of IL-1$\beta$ by this fungal pathogen.

The importance of understanding the mechanisms responsible for the production of IL-1$\beta$ by *C. albicans* is underlined by the important role that IL-1$\beta$ plays in anti-*Candida* host defense [6]. It is known that IL-1$\beta$ is produced during infection with *C. albicans* [35]. Accordingly, we show that, after contact with *C. albicans*, human PBMCs up-regulate mRNA for IL-1$\beta$ and secrete mature IL-1$\beta$. To determine which receptors of human

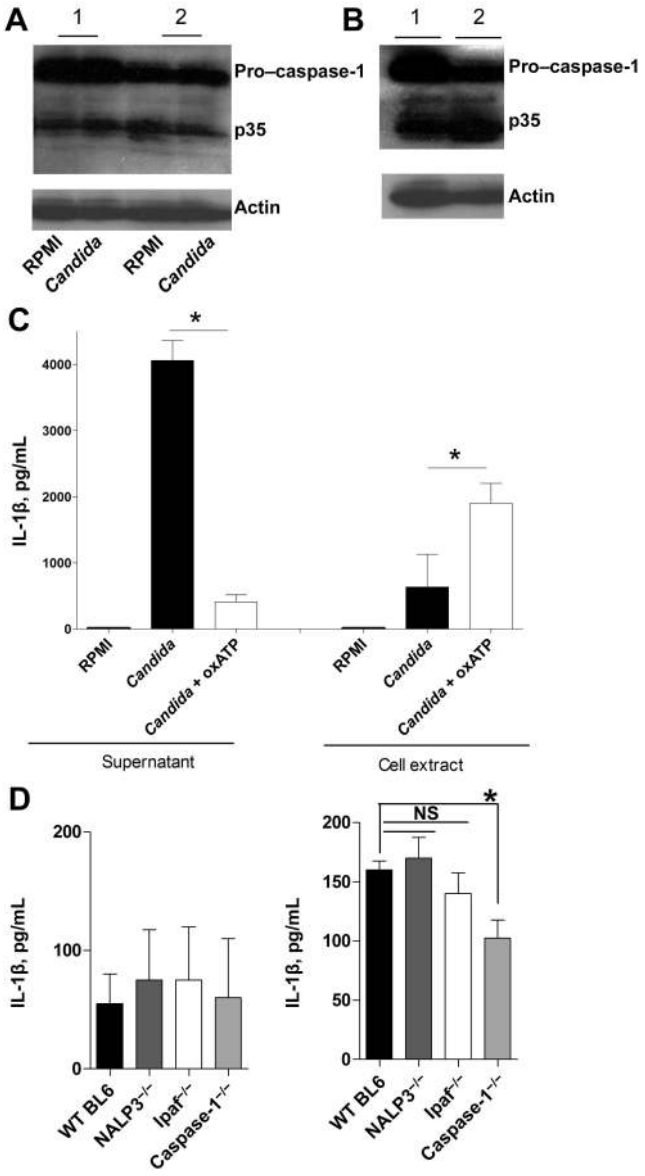


**Figure 3.** Constitutively active caspase-1 in human monocytes and adenosine-5'-triphosphate (ATP)–dependent interleukin (IL)–1$\beta$ secretion in human peripheral blood mononuclear cells (PBMCs). *A,* Western blot analysis for the detection of caspase-1 and $\beta$-actin in cellular lysates from PBMCs incubated with RPMI 1640 or *Candida albicans* at 37°C for 2 h. *B,* Western blot analysis for the detection of caspase-1 and $\beta$-actin in cellular lysates from unstimulated PBMCs directly lysed after isolation. *C,* Human PBMCs were stimulated with heat-killed *C. albicans* yeast cells ($10^6$ microorganisms/mL) in the presence or absence of the P2X7 receptor inhibitor oxATP. Production of IL-1$\beta$ in the supernatants was measured by ELISA after stimulation at 37°C for 24 h. Data are pooled from 2 separate experiments involving a total of 5 healthy volunteers (median $\pm$ interquartile range [IQR]; $^*P < .05$, by Wilcoxon rank test). *D,* In vitro IL-1$\beta$ production by resident peritoneal macrophages ($10^5$ microorganisms/mL) stimulated with heat-killed *C. albicans* ($10^7$ microorganisms/mL). The data in panel *D* are pooled from 2 separate experiments involving a total of 8 mice (median $\pm$ IQR; $^*P < .05$, by Mann-Whitney *U* test). Ipaf, IL-1 converting enzyme protease-activating factor; NALP3, cryopirin; NS, nonsignificant; WT BL6, wild-type C57BL/6.

PBMCs and components of *C. albicans* are responsible for these developments, receptor inhibitors and *C. albicans* cell wall mutants were used. The outer layer of the *C. albicans* cell wall is enriched with mannoproteins, whereas the inner layer is composed of chitin and $\beta$1,3- and $\beta$1,6-glucan. Recently, we and other investigators have shown that mannoproteins, phospholipomannan, and $\beta$-glucans are involved in cytokine stimulation induced by *C. albicans* [36–39]. Recognition of mannosyl residues is mediated by MR binding to *N*-linked mannosyl residues and by TLR4 binding to *O*-linked mannosyl residues [40]. A second pathway of cytokine production is mediated by the recognition of $\beta$-glucan through the dectin-1/TLR2 receptor complex. This has been demonstrated for TNF-$\alpha$ and IL-6, in which transcriptional regulation of cytokine production is very important. We demonstrated that stimulation of monocytes with *C. albicans* lacking *N*-linked mannosyl residues or blockade of the MR leads to a reduction in IL-1$\beta$ production. In addition, chitin has also been reported to be recognized by MR [41]. When chitin-binding sites were blocked, IL-1$\beta$ production by *C. albicans* was significantly impaired.

The lectin receptor dectin-1 recognizes $\beta$-glucan and interacts with TLR2 [42, 43]. TLR2 and dectin-1 are also involved in IL-1$\beta$ production after activation by *C. albicans* yeast cells in human monocytes. Interestingly, TLR2, but not dectin-1, is involved in the induction of IL-1$\beta$ by pseudohyphae, which is in line with the observation that *C. albicans* is able to completely mask its $\beta$-glucans after transition from the yeast to the hyphal form [34]. It is tempting to speculate that the loss of the additional effect of dectin-1 on TLR2 explains the lower amount of pseudohyphae-induced IL-1$\beta$ production. Syk tyrosine kinase, which can be phosphorylated by the immunoreceptor tyrosine-based activation motif of dectin-1 [44], is also involved in the induction of IL-1$\beta$.

In contrast, TLR4 is not involved in IL-1$\beta$ production induced by *C. albicans,* because neither blocking of TLR4 or CD14 nor stimulation with the *mnt1*$\Delta$ *mnt2*$\Delta$ double null mutant lacking the terminal 4 *O*-linked $\alpha$1,2-mannosyl residues that bind to TLR4 [29] altered IL-1$\beta$ production. This observation is surprising, considering that (1) *C. albicans* possesses known TLR4 ligands, such as *O*-linked mannosyl residues, that induce production of such proinflammatory cytokines as TNF-$\alpha$, and (2) LPS leads to potent IL-1$\beta$ production after contact with human monocytes, suggesting that TLR4 is involved in induction of IL-1$\beta$ [45]. These findings show that redundant mechanisms are responsible for IL-1$\beta$ stimulation by *C. albicans,* and they underline the principle that specific receptors can induce transcription of different subsets of genes in response to the same ligand. This results in the ability of immune cells to induce many different cytokine responses from a limited repertoire of receptors, according to the specific PAMPs that are present, and it helps to explain how the immune system induces tailored responses to specific pathogens. Similarly, phosphomannan is not involved.

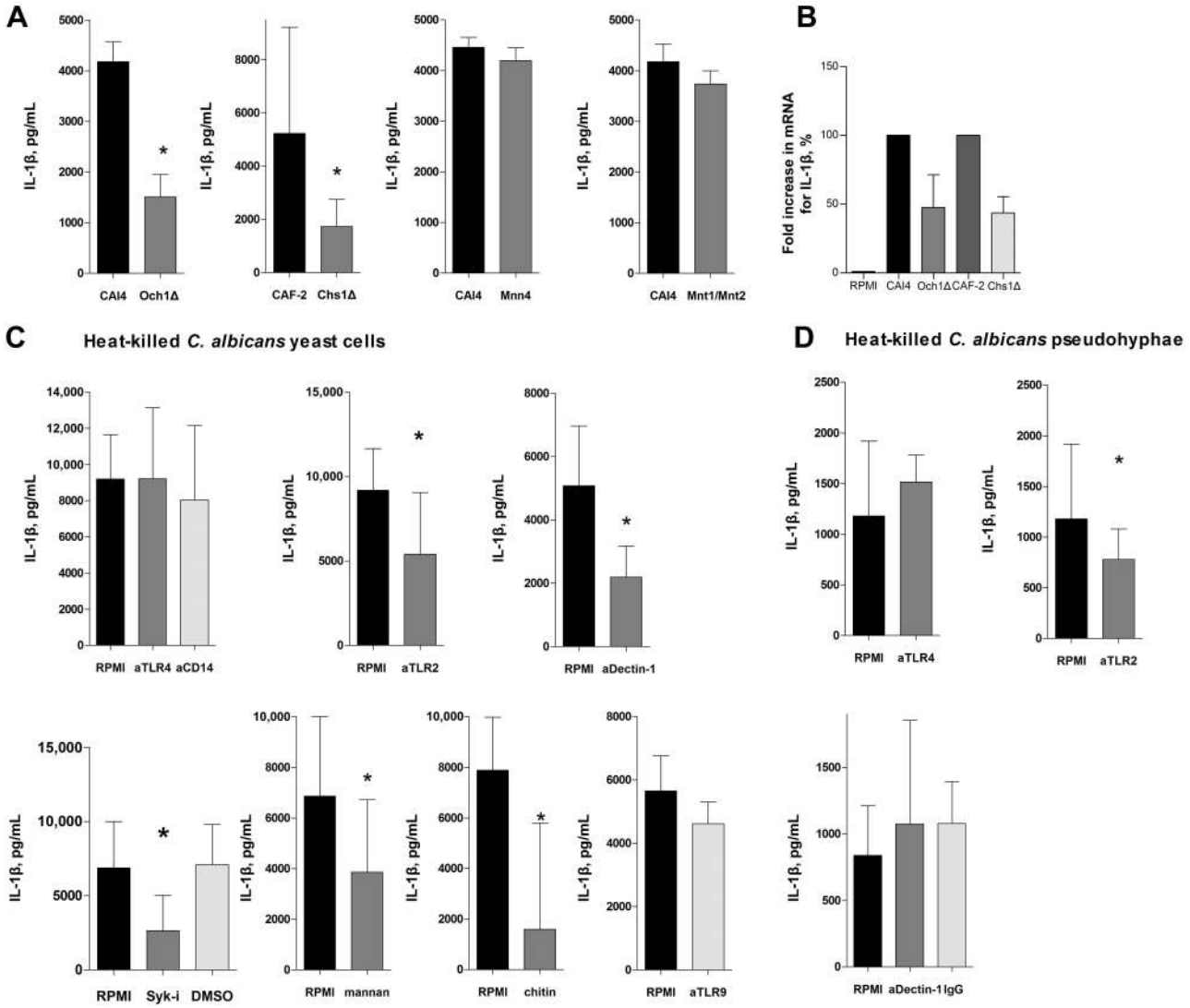


**Figure 4.** Dependence of interleukin (IL)–1$\beta$ production on Toll-like receptor (TLR) 2, dectin-1, mannose receptor, and chitin pathways. *A,* Human peripheral blood mononuclear cells (PBMCs) were stimulated with heat-killed wild-type *Candida albicans* CAI4 or CAF2 and null mutants affected in cell wall glycosylation at a concentration of $10^6$ microorganisms/mL for 24 h. IL-1$\beta$ concentrations were measured by ELISA. *B,* Reverse-transcriptase quantitative polymerase chain reaction analysis of mRNA for IL-1$\beta$ in PBMCs stimulated with *C. albicans chs1*$\Delta$ and *och1*$\Delta$ null mutants and their wild types. Human PBMCs were stimulated with heat-killed *C. albicans* yeast cells *(C)* or heat-killed pseudohyphae ($10^6$ microorganisms/mL) *(D)* in the presence or absence of specific receptor inhibitors (anti–TLR4 antibody [aTLR4], 20 $\mu$g/mL; anti-TLR2 antibody [aTLR2], 20 $\mu$g/mL; anti-CD14 WT14 antibody [aCD14], 20 $\mu$g/mL; and antibody to the dectin-1 receptor [aDectin-1], 10 $\mu$g/mL), mannan (200 $\mu$g/mL), or chitin (20 $\mu$g/mL) or Syk inhibitor (50 nmol/L). Production of IL-1$\beta$ in the supernatants was measured by ELISA after stimulation at 37°C for 24 h. Data are pooled from at least 2 separate experiments involving a total of at least 5 healthy volunteers (median $\pm$ interquartile range; $^*P < .05$, by Wilcoxon rank test). DMSO, dimethyl sulfoxide; Syk-i, Syk inhibitor.

One has to acknowledge differences in the stimulation of cytokines—in this case, IL-1$\beta$—by live or dead *Candida* organisms. Both of these forms of *Candida* organisms stimulated IL-1$\beta$. However, exposure of $\beta$-glucans at the cell wall surface is more prominent when yeasts are heat killed [34]. It is likely that the role of dectin-1 in IL-1$\beta$ induction is more prominent when heat-killed *Candida* organisms are used instead of live yeasts.

It has been suggested that processing of IL-1$\beta$ is mediated by caspase-1 activation by the "inflammasome," a protein complex formed mainly by receptors of the NLR family [12]. Several inflammasomes activate IL-1$\beta$ during bacterial infections, including NALP3 [20] and Ipaf inflammasomes [46]. Of note, no component of *C. albicans* has yet been reported to be able to interact with NLR receptors. We demonstrated that caspase-1 is the main enzyme that processes IL-1$\beta$ produced by human monocytes stimulated with *C. albicans.* However, the inflammasome components NALP3 and Ipaf were not needed for *Candida*-induced production of IL-1$\beta$. The fact that we observed a reduction in mRNA transcription for IL-1$\beta$ in PBMCs treated with a caspase-1 inhibitor can be explained by the known feedback

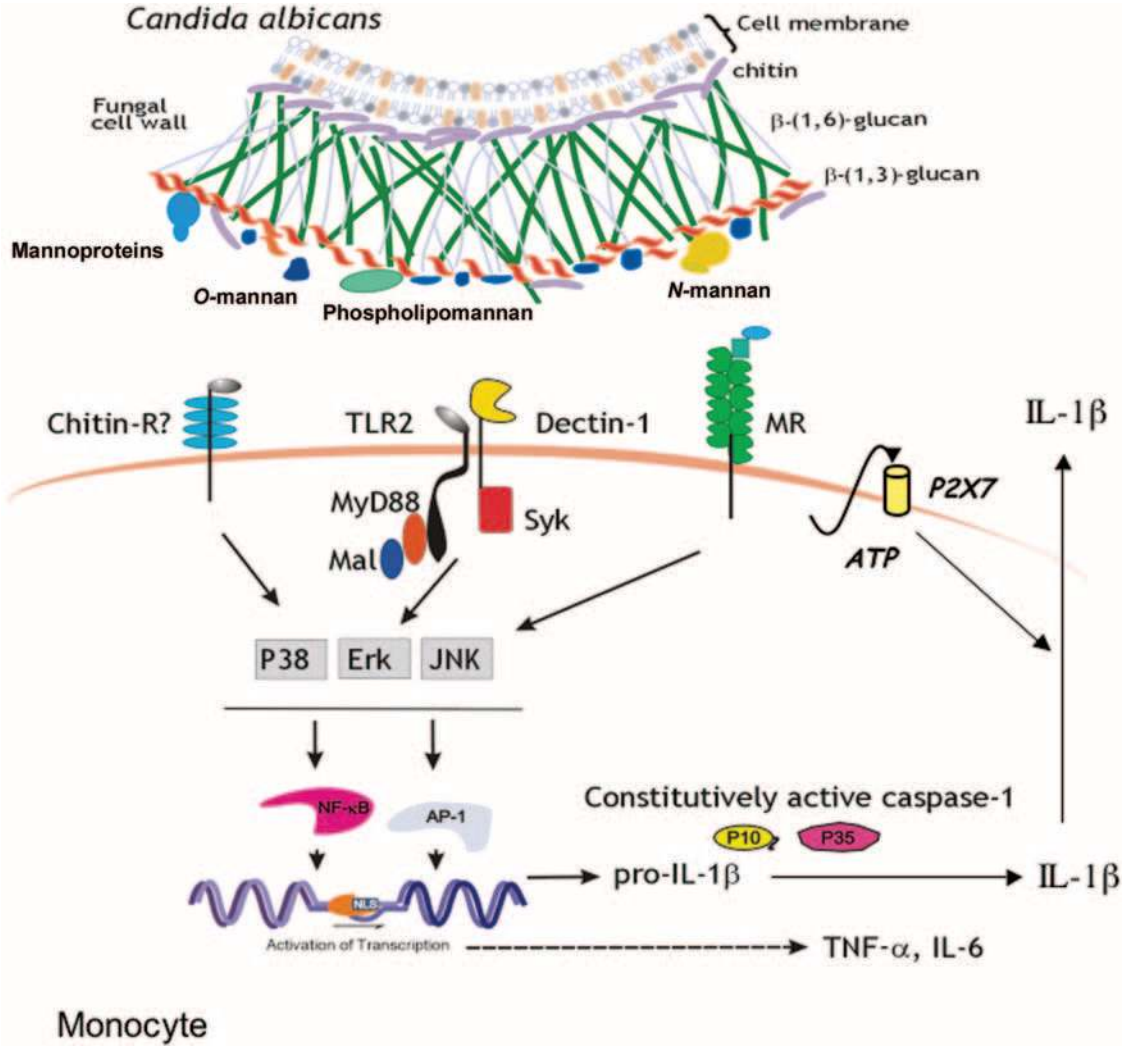


**Figure 5.** Pathway of activation of interleukin (IL)–1$\beta$ by *Candida albicans* in human monocytes. Schematic representation of the induction and signaling pathway of IL-1$\beta$ induced by the fungal pathogen *C. albicans* in the human primary monocyte. Induction involves a dectin-1/Toll-like receptor (TLR) 2 complex that recognizes $\beta$-glucan, as well as mannose receptor (MR) recognizing *N*-linked mannan and chitin, leading to transcription of mRNA for IL-1$\beta$. There is a possibility that chitin induces cytokines through other receptors, but this has not yet been confirmed. The dectin-1 signaling pathway is dependent on Syk and also induces transcription of mRNA for pro-IL-1$\beta$. Pro-IL-1$\beta$, in its turn, is cleaved by active caspase-1, which is constitutively activated in human monocytes, resulting in IL-1$\beta$ processing and secretion. The end result of this cascade is production of bioactive endogenous IL-1$\beta$. AP-1, activator protein-1; JNK, Jun N-terminal kinase; TNF, tumor necrosis factor.

loop of IL-1$\beta$ transcriptional induction and confirms that the endogenous IL-1$\beta$ production is bioactive [33]. Furthermore, we observed that caspase-1 was already present in its active form in freshly isolated human PBMCs, and p35 was not up-regulated by *C. albicans*. This observation suggests that inflammasome activation in human monocytes is not an important regulatory step in IL-1$\beta$ synthesis and release after stimulation with *C. albicans,* and it shows that, similar to IL-6 and TNF-$\alpha$ production, IL-1$\beta$ production by monocytes encountering *C. albicans* is mainly regulated at the level of transcription.

In addition to caspase-1, the serine protease PR3 has also been proposed to be able to process pro—IL-1$\beta$ [47]. Our data show that PR3 has an additional effect, albeit a relatively limited one, in the processing of IL-1$\beta$. Other serine proteases, such as cathepsin G and elastase, can play similar additional roles. This likely explains the residual IL-1$\beta$ production observed after maximal caspase-1 inhibition.

Although secretion of IL-1$\beta$ is incompletely understood, evidence points to the P2X7 receptor, a membrane receptor for extracellular ATP, as a key player in this process [48]. In line with this evidence, we found that blocking the P2X7 receptor leads to a reduction in IL-1$\beta$ secretion by stimulated monocytes. In addition, it has been reported that monocytes can release endogenous ATP [49]. We also observed that monocytes could release endogenous ATP and thus were able to provide their own signal for IL-1$\beta$ secretion.

In conclusion, we dissected the molecular mechanisms responsible for production of IL-1$\beta$ by a fungal pathogen. Several important pathways are involved in these processes: *C. albicans* activates IL-1$\beta$ through *N*-mannan and chitin recognition by MR, and $\beta$-glucan recognition by dectin-1/TLR2. It has also been suggested by Reese et al. [50] that chitin plays an important role as a PAMP in the innate immune system; however, whether receptors other than MR can recognize chitin remains to be elucidated. The tyrosine kinase Syk plays a role in the intracellular pathway inducing IL-1$\beta$ gene activation (see the diagram presented in figure 5). We demonstrated that caspase-1 activation is constitutive and does not need recognition of *C. albicans* by the inflammasome in human primary mononuclear cells. These data question the role of the inflammasome in the induction of IL-1$\beta$ by fungal pathogens. A deeper knowledge of the mechanisms of cell activation by *Candida* organisms may represent the basis for the future design of immunotherapeutic strategies in fungal infections.

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