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(1,3)-β-Glucans Activate Both Dectin-1 and NLRP3 Inflammasome in Human Macrophages

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β-glucans are naturally occurring polysaccharides that are the major cell wall components of fungi. Recognition of β-glucans is mediated through a membrane-bound pattern recognition receptor called dectin-1, and gene knock-out studies have shown that dectin-1 plays an important role in antifungal immune response in vivo. In this report, we have studied the effect of large particulate (1,3)-β-glucans, including curdlan, glucan from baker's yeast, paramylon, and zymosan, on inflammatory response in human macrophages. We show that β-glucans activate the transcription of the proinflammatory cytokine IL-1β through a dectin-1–dependent pathway in human macrophages. Moreover, dectin-1 receptor associated Syk tyrosine kinase was essential for β-glucan induced IL-1β mRNA expression. In contrast to LPS, β-glucans also strongly activated the secretion of IL-1β. This β-glucan triggered IL-1β release was abolished by cytochalasin D, an inhibitor of phagocytosis, demonstrating that cytosolic recognition of β-glucans is required for IL-1β response in human macrophages. RNA interference-mediated gene knockdown experiments demonstrated that cytoplasmic NLRP3 inflammasome is essential for β-glucan–induced IL-1β secretion. Moreover, our results suggest that β-glucan–induced NLRP3 inflammasome activation is dependent on the dectin-1/Syk signaling pathway. Furthermore, our results suggest that the lysosomal cathepsin B protease, the formation of reactive oxygen species, and the efflux of potassium are needed for β-glucan–induced NLRP3 inflammasome activation. In conclusion, our results show that β-glucans are recognized by membrane-associated dectin-1 and cytoplasmic NLRP3 inflammasome resulting in IL-1β gene transcription and IL-1β secretion in human macrophages, respectively. ***The Journal of Immunology*****, 2010, 184: 6335–6342.**

Our immune system recognizes and responds to infection through the pattern recognition receptors (PRRs) of the innate immune system (1). PRRs recognize highly conserved pathogen-associated molecular patterns (PAMPs) found in micro-organisms. For example, LPS, an endotoxin associated in cell walls of many Gram-negative bacteria, and β-glucan, a major fungal cell wall component, are PAMPs that are recognized by plasma membrane-bound PRRs called TLR4 and dectin-1, respectively. In addition to PAMPs, different heterogeneous danger associated molecular patterns (DAMPs) (2), including ATP and monosodium uric acid (MSU), enhance inflammatory response (3). Effective PAMP and DAMP recognition results in the activation of antimicrobial and inflammatory response in APCs, including macrophages and dendritic cells (DCs), leading to the activation of adaptive immune response and ultimately results in the eradication of the pathogen (4).

IL-1β is a proinflammatory cytokine that is critical for local and systemic inflammation (5). IL-1β is mainly produced by tissue macrophages and is kept strictly silent in normal circumstances by controlling the synthesis, secretion, and activity of the cytokine (5). Production of the biologically active form of IL-1β requires concerted action of membrane-bound and cytosolic PRRs (5). First, IL-1β gene expression has to be activated by membrane-bound PRRs, such as TLRs. After this, the transcribed immature form of IL-1β, proIL-1β, has to be cleaved by cysteine protease caspase-1 to generate the secreted, biologically active form of IL-1β (6). In parallel, caspase-1 is activated by cytosolic PRRs in a protein complex called inflammasome that acts as a molecular platform for the activation of caspase-1 (7). After the activation, caspase-1 processes proIL-1β to the biologically active form of IL-1β, which is readily secreted. The inflammasomes consist of an adapter molecule called apoptosis-associated speck-like protein containing a CARD and a PRR that belongs to either the NOD (nucleotide-binding oligomerization domain)-like receptor (NLR) or the PYRIN receptor gene family (8). Caspase-1 activating structures include NLRP3 (7, 9), NOD2/NLRP1 (10), NOD2/NLRP3 (11), IPAF/NAIP5 (12–16), and AIM2 inflammasomes (17–20). NOD2/NLRP1 and IPAF/NAIP5 inflammasomes are activated by cytosolic muramyl peptide (MDP) and bacterial flagellin, respectively (8). The most recently described AIM2 inflammasome is activated by cytosolic DNA (21). In contrast to NOD2/NLRP1, IPAF/NAIP5, and AIM2 inflammasomes, which are triggered by a limited number of conserved microbial ligands, NLRP3 inflammasome is activated by various heterogeneous DAMPs, including extracellular ATP, MSU, and hyaluronan (8, 22). In addition to these endogenous stimuli, NLRP3 inflammasome is also activated by exogenous stimuli, including asbestos, silica, aluminum adjuvant, and microbial toxins (8).

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Abbreviations used in this paper: AIM2, absent in melanoma 2; BHA, butylated hydroxyanisole; DAMP, danger associated molecular pattern; DC, dendritic cell; GBY, glucan from Baker's yeast; MDP, muramyl dipeptide; MSU, monosodium uric acid; NLR, NOD-like receptor; NOD, nucleotide-binding oligomerization domain; PAMP, pathogen-associated molecular patterns; PRR, pattern recognition receptor; ROS, reactive oxygen species; siRNA, small interfering RNA; ZYM, zymosan.

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β-glucans are naturally occurring carbohydrates that possess immunostimulatory activity. They form a heterogeneous group of polysaccharides, and their immunological activity depends on the molecular structure, including polymer length and degree of branching. In fungal cell walls, β-glucans occur as (1,3)-β-linked glucose polymer with varying length and number of (1,6)-β-glucose side chains (23). They are the major structural components of fungal cell wall and are therefore potentially important PAMPs for the PRR-mediated sensing of fungal infection. Indeed, dectin-1 is the major PRR for β-glucans (24–26). Triggering of dectin-1 by β-glucans activates phagocytosis, respiratory burst, and the production of cytokines and chemokines in macrophages and DCs (27). In general, large particulate β-glucans, such as curdlan and zymosan (ZYM), are the most potent β-glucans that trigger dectin-1 and have antimicrobial and immunomodulatory properties both in vitro and in vivo (23). In the current work, we have studied the mechanism of proinflammatory response triggered by large particulate (1,3)-β-glucans, including curdlan, paramylon, glucan from baker's yeast (GBY), and ZYM, in human primary macrophages. We demonstrate that the β-glucans are sensed by sophisticated cooperating pathways through both membrane-bound and cytosolic PRRs, resulting in robust activation of IL-1β–mediated inflammatory response in human primary macrophages.

Materials and Methods

Cells, Abs, and reagents

PBMCs from healthy blood donors (Finnish Red Cross Blood Transfusion Service, Helsinki, Finland) were isolated by low-speed density gradient centrifugation over Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden) as previously described (28). Monocytes were purified by adherence and were allowed to differentiate into macrophages by maintenance in the macrophage serum-free medium (Life Technologies, Paisley, U.K.) supplemented with 10 ng/ml GM-CSF (Biosource International, Camarillo, CA) and antibiotics, including 0.6 μg/ml penicillin and 60 μg/ml streptomycin (Life Technologies), in 6-well or 12-well plates. After 7 d of culture, the resulting macrophages were used in experiments. Optimal concentrations for LPS (*Escherichia coli* 0111: B4), curdlan, GBY, paramylon, and ZYM (all purchased from Sigma-Aldrich, St. Louis, MO) were pretitrated in preliminary experiments and the final amounts used in experiments for LPS was 0.1 μg/ml, for all the β-glucans 10 μg/ml, and 100 μg/ml. Abs and inhibitors used in the experiments were anti-human dectin-1 mAb (9 μg/ml; R&D Systems, Minneapolis, MN), Syk tyrosine kinase inhibitor II (1 μM; Calbiochem, San Diego, CA), human caspase-1 inhibitor, Z-YVAD-fmk (50 μM; Santa Cruz Biotechnology, Santa Cruz, CA), cytochalasin D (1 μg/ml; Sigma Aldrich), cathepsin B inhibitor, CA-074-Me (10 μM; Calbiochem), and butylated hydroxyanisole (BHA) (100 μM; Sigma-Aldrich). When Abs or inhibitors were used, they were added to macrophage serum-free media 0.5 h prior to stimulation with curdlan. Elevated potassium chloride (Sigma-Aldrich) concentration (1 mM supplementation) was added to the macrophage serum-free medium 0.5 h prior stimulation of the cells with curdlan.

Quantitative real-time RT-PCR assay and data analysis

Total cellular RNA was extracted using Trizol Reagent (Life Technologies, Carlsbad, CA). RNA concentration and purity were determined by spectrophotometer (GeneQuant Pro RNA/DNA calculator, Amersham Biosciences, Cambridge, U.K.) measuring absorbance at A_{260} and A_{280} nm. RNA was temporarily stored at −70°C in nuclease-free water. The 0.5 μg total RNA were reverse transcribed in a 25 μl reaction mixture using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) according to manufacturer's instructions. The cDNA was amplified in 11 μl of 1× TaqMan Fast universal PCR master mix with Predeveloped TaqMan assay primers and probes (IL-1β Hs0017497_m1, NLRP3 Hs_00918082_A1, Applied Biosystems) according to manufacturer's instruction. The primer and probe sets were designed and optimized according to Applied Biosystems guidelines. Quantification was performed by using the comparative $\Delta\Delta C_T$ method as previously described (29). Real-time PCR data were developed by using the Sequence detector system version 1.4 software (Applied Biosystems) and the analyzed results were finally expressed as relative units.

Western blotting and ELISA

IL-1β processing and secretion were analyzed by Western blot performed either by using whole-cell extract or by using concentrated cell supernatants. IL-1β processing was analyzed from whole-cell extract. Cells were lysed in protein lysis buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, and 25% ethylene glycol supplemented with complete mini protease inhibitor mixture (Roche Diagnostic, Indianapolis, IN) after which the extract was homogenized with ultrasound sonicator (Sanyo Electronics, San Diego, CA). We determined the total protein concentrations with Bio-Rad Dc Protein Assay (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. In parallel, IL-1β secretion was analyzed from concentrated cell supernatants. Cell culture supernatants (5 ml) were concentrated by Amicon UItra-15 tubes, (Millipore, Bedford, MA) according to the manufacturer's instructions. After isolation and homogenization of cell lysis extract or after concentration of cell culture supernatants, 10 μg of proteins from whole lysed cell extracts or 2.5 μl from 250 μl concentrated cell supernatants were separated on 15% SDS-PAGE at 200 V and transferred onto Immobilon-P Transfer Membranes (Millipore) by the Isophor electro transfer apparatus PowerPac Basic (Bio-Rad Laboratories) at +4°C and 100 V for 2 h. The membranes were blocked in PBS containing 5% nonfat milk for 45 min, after which they were incubated at +4°C overnight. After this, membranes were incubated at room temperature for 1 h with appropriate HRP-conjugated secondary Abs (Dako A/S, Glostrup, Denmark.). Finally, proteins were visualized by the ECL system (PerkinElmer Life Sciences, Zaventem, Belgium). To confirm equal loading and transfer of the protein samples, membranes were stripped and stained with ready-to-use SYPRO Ruby Protein Blot Stain according to the manufacturer's instructions (Sigma-Aldrich). The major protein band detected and basally expressed is shown as a loading control in each Western blot analysis. Anti–IL-1β Ab has previously been described (30, 31). Anti–caspase-1 p20 Ab was purchased from Sigma-Aldrich. Human IL-1β Eli-pair was purchased from Diaclone (Besançor Cedex, France) and human IL-18 ELISA from Bender MedSystems (Bender MedSystems, Austria) and ELISAs were performed according to the manufacturer's instructions.

Small interfering RNA assays

After 5 d of cell culture in 12-well plates, macrophages were transfected with 50 nM nontargeting control small interfering RNA (siRNA) (AllStars Negative Control siRNA, Qiagen, Valencia, CA) and with 25 nM of two different NLRP3 siRNAs (Hs_CIAS1_6, Hs_CIAS1_9; Qiagen) using the HiPerFect Transfection Reagent (Qiagen) according the manufacturer's instruction. The transfection was repeated the following day and after 24 h macrophages were left unstimulated or stimulated for 12 h with curdlan, and total cellular RNA was isolated and cell culture supernatants were collected.

Statistical analyses

Each macrophage sample represents a pool of separately stimulated cells from three different blood donors. RT-PCR and ELISA results are combined through three different stimulations and Western blot results are representative of three independent, but similarly performed experiments unless otherwise mentioned. Data were analyzed using the GraphPad Prism 4 Software (GraphPad Software, San Diego, CA). An unpaired *t* test or Mann-Whitney *U* test was used to compare the differences between the groups. A p value of <0.05 was considered to be statistically significant. In RT-PCR and ELISA figures, data were expressed as means (± SD).

Results

(1,3)-β-glucans activate transcription and secretion of IL-1β in human macrophages

Fungal β-glucans have both antimicrobial and immunomodulatory effects (23). To compare β-glucan and LPS-induced proinflammatory cytokine response in human cells, macrophages were stimulated for different periods with LPS and the large particulate (1,3)-β-glucan, curdlan. After this, the cells were collected, total cellular RNA was extracted, and IL-1β mRNA expression was studied by quantitative RT-PCR. Both curdlan and LPS strongly activated transcription of the IL-1β gene (Fig. 1*A*). LPS-triggered IL-1β mRNA expression was highest at 2 h after stimulation, whereas curdlan-induced IL-1β mRNA expression peaked at 6 h after stimulation (Fig. 1*A*).

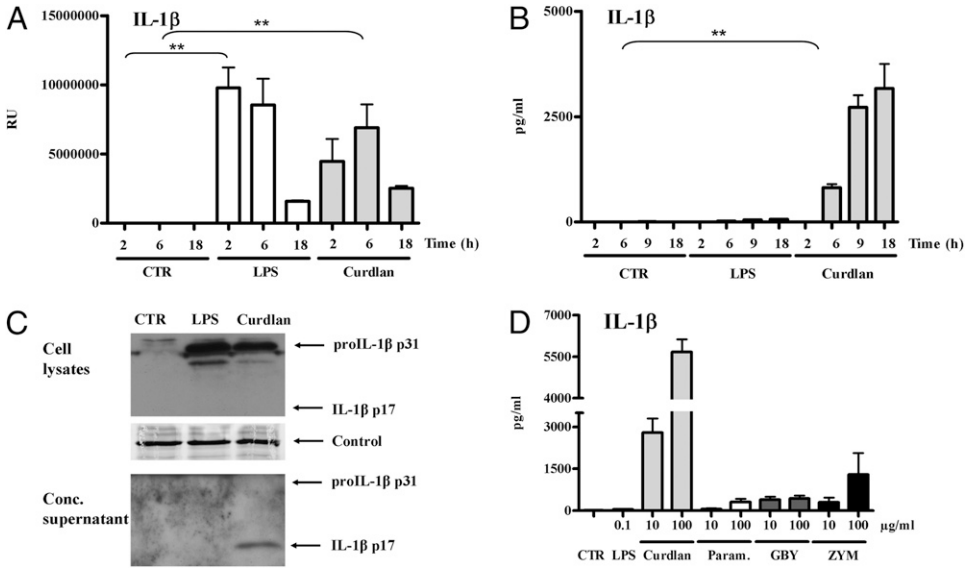

FIGURE 1. Large particulate (1,3)-β-glucans activate transcription and secretion of IL-1β in human macrophages. *A*, Human macrophages were incubated at the times indicated with curdlan (10 μg/ml) and LPS (0.1 μg/ml). After this, the cells were harvested, total cellular RNA was extracted, cDNA was synthesized, and IL-1β mRNA expression was analyzed by quantitative RT-PCR. The RT-PCR data represents relative units (RU), that is, fold change in gene expression, which is normalized to an endogenous reference gene (18S rRNA) and is relative to a no-template control calibrator. *B*, Human macrophages were incubated at the times indicated with 10 μg/ml curdlan and 0.1 μg/ml LPS. After this cell culture supernatants were collected and IL-1β secretion analyzed by ELISA. *C*, The processing and secretion of IL-1β was verified by Western blot analysis. Human macrophages were stimulated for 18 h with 10 μg/ml curdlan and 0.1 μg/ml LPS. The cells were harvested and protein lysates were prepared (*top panel* as indicated) or cell culture supernatants were concentrated (*lower panel* as indicated) and IL-1β expression was analyzed by Western blotting with anti–IL-1β Abs. To confirm equal loading and transfer of the protein samples, membranes were stripped and stained with ready-to-use SYPRO Ruby Protein blot stain. The major protein band detected and basally expressed is shown as a loading control in Western blot analysis (indicated as control). *D*, Human macrophages were stimulated for 18 h with curdlan, GBY, paramylon, and ZYM as indicated. Secreted IL-1β was analyzed from cell culture supernatants by ELISA. *A, B*, and *D*, Values are means ± SD from three independent analyses. $**p < 0.01$. *C*, Each macrophage sample represents a pool of separately stimulated cells from three different blood donors and the results are representative of three independent, but similarly performed experiments.

The transcribed immature form of IL-1β, pro–IL-1β, has to be cleaved by cysteine protease caspase-1 to activate secretion of the biologically active form of IL-1β (6). To analyze whether stimulation with the microbial cell wall components, β-glucan and LPS, results in the formation of the mature form of IL-1β in human macrophages, we activated the cells with curdlan and LPS for different periods and IL-1β secretion was determined from cell culture supernatants. Our results show that LPS elicited very little release of IL-1β. In contrast to LPS, curdlan strongly activated secretion of IL-1β in human macrophages already 6 h after stimulation and maximal levels of IL-1β were detected at 18 h poststimulation (Fig. 1*B*). To verify the production and processing of pro–IL-1β in the cells and to confirm the secretion of the mature form of IL-1β into the cell culture supernatants, we stimulated macrophages with curdlan and LPS for 18 h and performed Western blot analysis from cell lysates and concentrated cell culture supernatants (Fig. 1*C*). Both curdlan and LPS activated the formation of the immature 31 kDa form of IL-1β (pro–IL-1β) in macrophages. However, only curdlan was able to induce the appearance of the biologically active 17 kDa form of IL-1β in cell culture supernatants (Fig. 1*C*). The data suggest that curdlan triggers both the transcription and secretion of IL-1β in human macrophages.

Like curdlan, GBY, paramylon, and ZYM, are large particulate (1,3)-β-glucans with immunostimulatory properties. To analyze whether paramylon, GBY, and ZYM can stimulate IL-1β secretion, we performed ELISA analysis from the cell culture supernatants of macrophages stimulated with these β-glucans. Paramylon, GBY, and ZYM elicited IL-1β secretion albeit at lower level compared with curdlan (Fig. 1*D*). In addition, in our experiments IL-1β secretion was also induced by β-glucans, including curdlan and ZYM, in human primary DCs (Supplemental Fig. 1). Thus, our results show that different (1,3)-β-glucans can activate IL-1β production and secretion in human primary macrophages and DCs. In additional experiments, curdlan was used as a representative (1,3)-β-glucan.

β-glucan induced IL-1β mRNA expression is dependent on dectin-1/Syk signaling pathway

Dectin-1 is the major immunostimulatory receptor described for β-glucans, including curdlan (23). To confirm the role of the dectin-1 in β-glucan triggered proinflammatory response, human PBMC-derived macrophages were stimulated with curdlan in the presence and absence of anti–dectin-1 Ab for 6 h. The neutralizing anti–dectin-1 Ab clearly inhibited both the transcription and secretion of IL-1β in response to curdlan stimulation showing that curdlan activates the transcription of the *IL-1β* gene through dectin-1 (Fig. 2*A*, 2*B*).

Dectin-1 has a short cytoplasmic tail, which contains ITAM -like sequences (32). ITAM-containing proteins signal through spleen tyrosine kinase (Syk) to activate gene expression (33) and Syk is also involved in dectin-1 signaling (34). To study the role of Syk in curdlan-induced IL-1β production, human macrophages were activated with curdlan for 6 h in the presence and absence of Syk inhibitor. As shown in Fig. 2*C* and 2*D*, the inhibition of Syk signaling significantly diminished IL-1β transcription and secretion in human macrophages. Collectively, these results demonstrate that β-glucans activate IL-1β transcription through the dectin-1– and Syk-dependent signaling pathway in human macrophages.

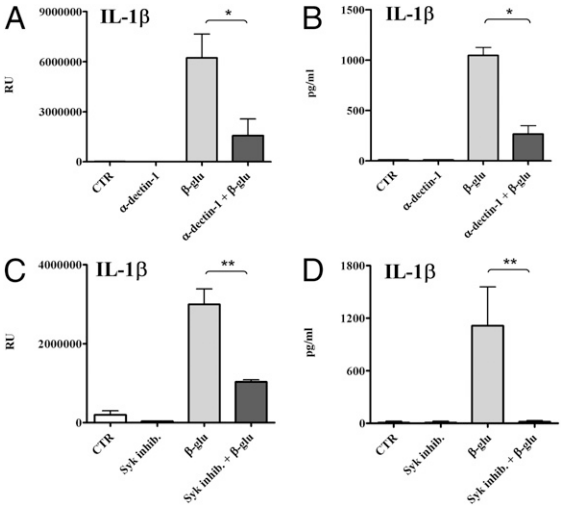

FIGURE 2. β-glucan activates IL-1β production through dectin-1- and Syk-dependent pathway. Human macrophages were incubated with (1,3)-β-glucan, curdlan for 6 h (10 μg/ml; β-glu) in the presence and absence of either anti–dectin-1 Ab or Syk inhibitor. *A* and *C*, Total cellular RNA was isolated and RT-PCR analysis was performed. Values are means ± SD from two independent experiments. *B* and *D*, Cell culture supernatants were harvested and IL-1β ELISA was performed. All values are means ± SD from three independent analyses. $*p < 0.05$; $**p < 0.01$.

Intracellular recognition of β-glucans is essential for IL-1β secretion

Previous studies have shown that the activation of cytoplasmic NLRP3 inflammasome in response to MSU and silica is dependent on the phagocytosis of these crystals (35). To study whether phagocytosis of curdlan is required for its ability to activate IL-1β secretion, macrophages were treated with cytochalasin D, a well-characterized inhibitor of phagocytosis, for 0.5 h after which the cells were left untreated or stimulated with curdlan for 6 h. Cytochalasin D had little effect on IL-1β transcription activated by the β-glucan (Fig. 3*A*). However, cytochalasin D completely abrogated IL-1β release triggered by curdlan (Fig. 3*B*). The results indicate that, in addition to membrane-bound dectin-1, β-glucans are also sensed by cytoplasmic PRRs resulting in IL-1β secretion in human macrophages.

β-glucan–induced IL-1β secretion is dependent on NLRP3 inflammasome

The NLRP3 inflammasome is activated by many PAMPs and endogenous DAMPs (8). To study whether β-glucans can activate NLPR3 inflammasome, we performed gene silencing experiments with human *NLRP3* gene sequence-specific siRNAs in human macrophages. The silencing of NLPR3 mRNA expression was successfully verified by quantitative RT-PCR (Fig. 4*A*). Moreover, as we expected, the *NLRP3* gene-silencing had little effect on the β-glucan–induced IL-1β mRNA expression (Fig. 4*B*). However, the NLRP3 siRNA treatment clearly decreased IL-1β secretion in response to the β-glucan stimulation suggesting that β-glucans trigger NLRP3 inflammasome in human macrophages (Fig. 4*C*). Recent reports have shown that NF-κB–dependent upregulation of NLRP3 expression is essential for the inflammasome activation (36, 37). Therefore, we analyzed by quantitative RT-PCR whether β-glucans upregulate NLRP3 gene expression in human macrophages. Both LPS and β-glucan stimulation led to NLRP3 mRNA induction in a time-dependent manner (Fig. 4*D*).

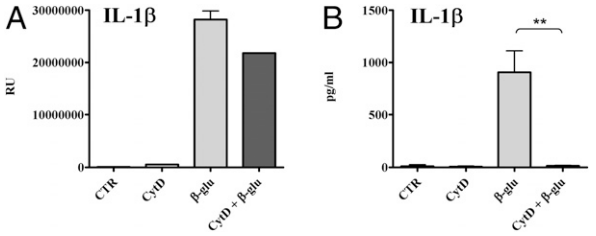

FIGURE 3. Cytochalasin D inhibits β-glucan–induced IL-1β secretion. Macrophages were treated with cytochalasin D (1 μg/ml; CytD) or left untreated after which curdlan (10 μg/ml; β-glu) was added and incubated for 6 h. *A*, The cells were collected for IL-1β quantitative mRNA analysis. Each macrophage sample represents a pool of separately stimulated cells from three different blood donors and the results are representative of three independent, but similarly performed experiments. Values are means ± SD from duplicate sample analyses from a single experiment. *B*, Cell culture supernatants were collected for IL-1β ELISA analysis. The means of three separate experiments (± SD) are shown. $**p < 0.01$.

Dectin-1/Syk signaling pathway is essential for inflammasome activation in human macrophages in response to β-glucan stimulation

Although the production of IL-1β typically requires an inducing stimulus, pro–IL-18 is constitutively expressed, and needs only processing by caspase-1 to be activated. Therefore, IL-18 secretion can be regarded as a direct marker of inflammasome activation. To study the role of the dectin-1 and Syk signaling in β-glucan–triggered inflammasome activation, macrophages were stimulated with curdlan in the presence and absence of anti–

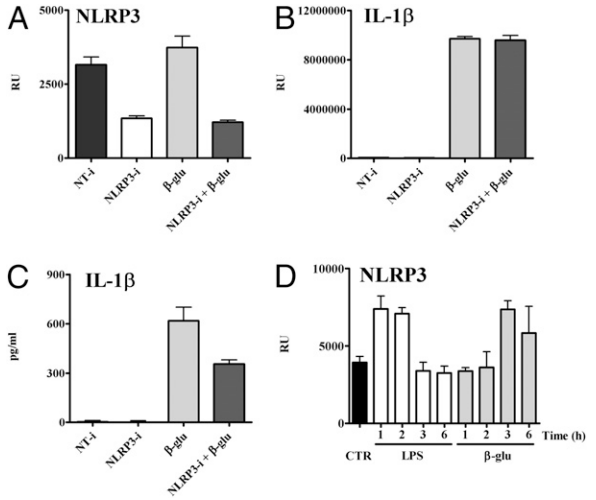

FIGURE 4. β-glucan–induced IL-1β secretion is dependent on NLRP3 inflammasome. Macrophages were transfected using the nontargeting siRNA control (NT-i) or the NLRP3 siRNAs (NLRP3-i) as described in *Materials and Methods*. Cells were both stimulated and left unstimulated for 12 h with curdlan. Each macrophage sample represents a pool of separately stimulated cells from three different blood donors and the results are representative of three independent, but similarly performed experiments. *A*, NLRP3-gene knockdown was verified from harvested cells by quantitative RT-PCR analysis of NLRP3. *B*, IL-1β mRNA expression was analyzed from cells by RT-PCR. *C*, Secretion of IL-1β was measured from cell culture supernatants by ELISA. Values are means ± SD from duplicate analyses from a single experiment. *D*, Human macrophages were stimulated with LPS (1 μg/ml) and/or curdlan (10 μg/ml; β-glu) for the times indicated. After this, the cells were harvested, total cellular RNA was prepared, cDNA was synthesized, and NLRP3 mRNA expression was analyzed by quantitative RT-PCR. Values are means ± SD from two independent experiments.

dectin-1 Abs and Syk inhibitor for 6 h and IL-18 secretion was studied by ELISA. Both anti–dectin-1 Abs and the Syk inhibitor completely abolished IL-18 secretion induced by β-glucan stimulation (Fig. 5).

Inhibition of caspase-1 and cathepsin B abolishes β-glucan–induced IL-1β release

Caspase-1 and cathepsin B are cysteine proteases, which are involved in activation of IL-1β release. Caspase-1 processes pro–IL-1β to the biologically active form of IL-1β (6). However, caspase-1–independent pathways for pro–IL-1β processing also have been described (5). In our study, curdlan activated formation of caspase-1 p20 in human macrophages (Fig. 6*A*). The activation was seen already at 3 h after exposure to the β-glucan. In contrast to curdlan, LPS did not activate caspase-1 p20 (Fig. 6*A*). Further, to confirm whether β-glucan–induced IL-1β secretion is dependent on caspase-1–mediated processing of pro–IL-1β, macrophages were activated with curdlan in the presence of a specific caspase-1 inhibitor, z-YVAD-fmk for 6 h. After this, the IL-1β mRNA expression and IL-1β secretion were studied. As expected, z-YVAD-fmk had little effect on IL-1β transcription (Fig. 6*B*). However, it strongly inhibited the curdlan-induced IL-1β secretion in human macrophages (Fig. 6*C*).

Lysosomal protease cathepsin B is known to have caspase-1 processing activity (38, 39). Currently, it has been suggested that the release of cathepsin B from the lysosomal compartment activates NLRP3 inflammasome by a yet uncharacterized mechanism. Phagocytosis of MSU, silica crystals, or aluminun adjuvants results in lysosomal release of cathepsin B release into the cell cytosol that is associated with NLRP3 activation (35). To study the role of cathepsin B in β-glucan–induced IL-1β secretion, macrophages were left untreated or stimulated with curdlan in both the presence and absence of a specific cathepsin B inhibitor, CA-074-Me for 6 h. CA-074-Me did not significantly effect IL-1β transcription induced by curdlan (Fig. 6*D*). In contrast, the cathepsin B inhibitor completely abolished the secretion of IL-1β in curdlan-stimulated macrophages (Fig. 6*E*).

Reactive oxygen species formation and potassium efflux are essential for β-glucan–triggered IL-1β secretion

Microbial toxins and crystalloid substances trigger reactive oxygen species (ROS) formation, which has been suggested to be a key event in NLRP3 inflammasome activation (8). Therefore, we analyzed whether ROS formation is required for β-glucan–induced IL-1β release in human macrophages. BHA, a known ROS inhibitor, completely abrogated curdlan-induced IL-1β release indicating that ROS formation is essential for β-glucan–triggered IL-1β release in human macrophages (Fig. 7*B*). BHA-treatment had only little effect on curdlan-induced IL-1β mRNA expression (Fig. 7*A*).

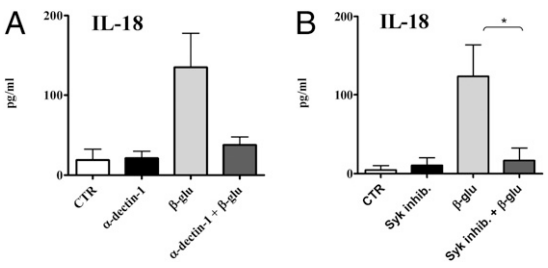

FIGURE 5. Dectin-1/Syk signaling pathway is essential for inflammasome activation in response to β-glucan stimulation. *A* and *B*, Macrophages were treated with LPS (1 μg/ml) and/or curdlan (10 μg/ml; β-glu) for 6 h in the presence and absence of anti–dectin-1 Abs or Syk inhibitor. After this, the cell culture supernatants were collected and IL-18 secretion was analyzed by ELISA. Values are means ± SD from three independent analyses. $*p < 0.05$.

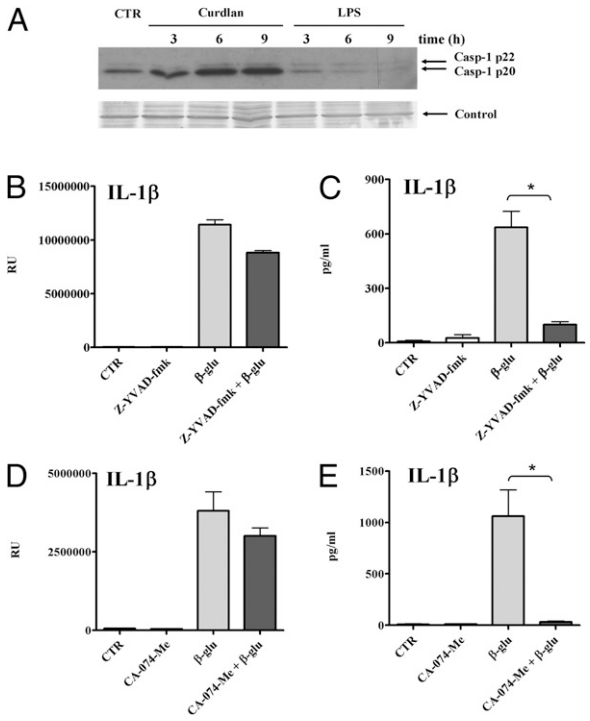

FIGURE 6. β-glucan–induced IL-1β secretion is dependent on caspase-1 and cathepsin B. *A,* Macrophages were stimulated with curdlan (10 μg/ml; β-glu) and LPS (0.1 μg/ml) for the times indicated. After this the cells were collected and protein lysates were prepared. Equal amounts of proteins (10 μg) were separated on 15% SDS-PAGE, and transferred on membranes that were stained with anticaspase-1 p20 Abs, and subsequently subjected to ECL. Macrophages were stimulated with curdlan (10 μg/ml; β-glu) in the presence and absence of either caspase-1 inhibitor, z-YVAD-fmk (50 μM) or cathepsin B inhibitor, CA-074-Me (10 μM) for 6 h. *B* and *D,* Cells were collected and used for IL-1β mRNA expression analysis by quantitative RT-PCR. The results are representative of three independent, but similarly performed experiments. Values are means ± SD from duplicate sample analyses from a single experiment. *C* and *E*, Harvested cell culture supernatants were used for IL-1β ELISA analysis. Values are means ± SD from three independent analyses. $*p < 0.05$; $**p < 0.01$.

In addition to ROS, potassium efflux has been shown to facilitate NLRP3 inflammasome activation in response to a variety of danger signals (40). To study the role of potassium efflux in β-glucan–triggered IL-1β secretion, human macrophages were stimulated with curdlan in the presence and absence of additional potassium chloride as described in *Materials and Methods*. The enhanced potassium chloride treatment did not significantly affect curdlan-induced IL-1β gene transcription (Fig. 7*C*). However, it completely blocked curdlan-induced IL-1β release (Fig. 7*D*). These results suggest that potassium efflux is needed for β-glucan–induced inflammasome activation in macrophages.

Discussion

Opportunistic fungi can cause severe infections in immunocompromised persons. In parallel, exposure to fungal components is linked to symptoms seen in people with DAMP building syndromes. IL-1β, which is a central mediator of acute and chronic inflammation, is likely to play an important role in mediating adverse health effects in fungal exposures. However, PAMPs and

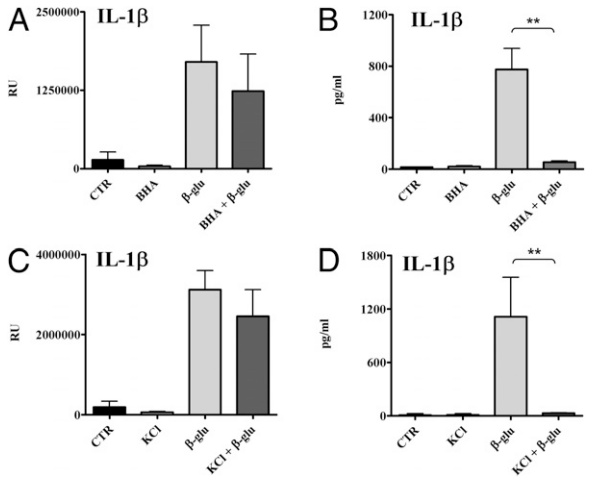

FIGURE 7. ROS production and potassium efflux are required for β-glucan–induced NLRP3 inflammasome activation. Human macrophages were treated or left untreated either with BHA (10 μM) or enhanced concentration of potassium chloride and further incubated with curdlan (10 μg/ml; β-glu) for 6 h. *A* and *C*, IL-1β mRNA expression was analyzed by quantitative RT-PCR. Values are means ± SD from two independent experiments. *B* and *D*, IL-1β secretion was analyzed from cell culture supernatants. The means of three separate experiments (± SD) are shown. $*p < 0.05$; $**p < 0.01$.

PRRs involved in triggering inflammatory response during fungal exposure have remained only partially characterized. In this report, we demonstrate that recognition of a major cell wall component of fungi, (1,3)-β-glucan, activates both the transcription and secretion of IL-1β through PRRs dectin-1 and NLRP3 inflammasome, respectively, in human macrophages.

In our experiments, (1,3)-β-glucans, including curdlan, GBY, paramylon, and ZYM, activated IL-1β secretion in human macrophages and DCs (Fig. 1*C*, Supplemental Fig. 1). Of those, curdlan and ZYM were the most potent inducers of IL-1β secretion. Both curdlan and ZYM are large (1,3)-β-glucans, but ZYM is also known to contain mannans and chitins, which can stimulate responses through TLRs (41, 42). Dectin-1 was the first characterized PRR for β-glucans and it is mainly expressed in APCs, including DCs and macrophages (24). Dectin-1 is a C-type lectin receptor that contains ITAMs in its cytoplasmic tail (32). On ligand binding, Syk tyrosine kinase is recruited to the dectin-1 receptor complex through ITAM interaction and activated (34). Our data show that both dectin-1 and Syk are essential for IL-1β transcription activated by β-glucans in human macrophages (Fig. 2). This is in line with previous genetic studies showing that Syk is an essential component of dectin-1 signaling pathway. It is also well documented that dectin-1–mediated fungal recognition preferentially induces IL-17–producing Th17 cells (43). This Th17 response is essential for antifungal defense and IL-1β has a crucial role in the development of Th17 response in human cells (44). Based on our results, it seems probable that IL-1β secretion induced by fungal β-glucans in human macrophages has a significant role in the development of the Th17 adaptive immune response.

Very recently, Gross and coworkers (45) demonstrated that NLRP3 inflammasome has an essential role in antifungal immunity. They showed that NLRP3-deficient mice are hypersusceptible to *Candida albicans* yeast infection. Our results suggest that β-glucans are the important fungal PAMPs that trigger innate immune response during fungal infection. They are recognized not only by membrane bound dectin-1 receptor, but also by cytoplasmic NLRP3 inflammasome. Gene silencing experiments with NLRP3-specific siRNA molecules treatment clearly decreased IL-1β secretion in response to the β-glucan stimulation (Fig. 4*C*) demonstrating that NLRP3 is also essential for inflammatory response activated by β-glucans in human macrophages.

The signaling pathways that lead to NLRP3 inflammasome activation are still poorly characterized. It was recently shown that the tyrosine kinase Syk, operating downstream of fungal pattern recognition receptor dectin-1, controls both pro–IL-1β synthesis and inflammasome activation after cell stimulation with *C. albicans* (45). Our results suggest that dectin-1/Syk signaling pathway is not only needed for the activation of IL-1β transcription (Fig. 2) but also for the inflammasome activation triggered by β-glucans (Fig. 5). It has been shown that NF-κB–dependent upregulation of NLRP3 expression is essential for the inflammasome activation (36, 37). Our data shows that NLRP3 gene expression is upregulated by β-glucan stimulation in human macrophages (Fig. 4*D*). It may be that dectin-1/Syk activated NF-κB induces *NLRP3* gene expression, which is required for the inflammasome activation. However, the Syk inhibitor abolished IL-18 secretion also in LPS-primed macrophages that express NLRP3 at high level (Supplemental Fig. 2) suggesting that Syk is required for NLRP3 inflammasome activation. Further studies are required to elucidate the phosphorylation targets of Syk that are essential for the inflammasome activation in response to β-glucan stimulation.

The hallmark of NLRP3 inflammasome activation is proteolytic processing of cysteine protease caspase-1. Activated caspase-1 in turn processes pro–IL-1β to the biologically active form of IL-1β. Consistent with this, the caspase-1 specific inhibitor, z-YVAD-fmk, clearly inhibited β-glucan–induced secretion of IL-1β (Fig. 6*C*). However, the molecular mechanisms leading to the activation of inflammasome-associated caspase-1 is mainly unclear. In general, inflammasomes respond to immunomodulatory PAMPs, including microbial peptidoglycans and nucleic acids (8). MDP, a peptidoglycan constituent of both Gram-positive and Gram-negative bacteria, is known to activate inflammasome-associated caspase-1 and IL-1β release. Genetic studies in mice have shown that IL-1β release induced by MDP requires both NLRP3 and NOD2 suggesting that multiple NLRs may cooperate to activate inflammatory response (11). Gene knock-out studies are required to define the essential components of NLRP3 inflammasome that are required for responses activated by β-glucans in vivo.

Many crystalline substances, including asbestos, silica, and uric acid, are potent activators of NLRP3 inflammasome. The phagocytosis of these crystals has been shown to be essential for the activation process (35, 46, 47). In our experiments, cytochalasin D, an inhibitor of phagocytosis, completely inhibited IL-1β release in response to β-glucan stimulation (Fig. 3). These data show that cytosolic recognition of β-glucans is needed for the inflammasome activation. Studies with silica and aluminum crystals have shown that the frustrated phagocytosis of these substances results in lysosomal swelling and rupture in macrophages. This lysosomal damage activates NLRP3 inflammasome through a cathepsin B-dependent pathway (35). Similarly, in our experiments with human primary macrophages cathepsin B-specific inhibitor completely abrogated IL-1β release triggered by β-glucans (Fig. 6*E*). Further studies are required to define the target molecules of cathepsin B that are involved in NLRP3 inflammasome activation.

NLRP3 inflammasome is activated by many microbial PAMPs and self-derived DAMPs, which are chemically and structurally different stimuli. This suggests that these stimuli are not directly recognized by the NLRP3 inflammasome. It is more likely that they induce inflammasome activation indirectly by activating production of endogenous danger signals. In addition to frustrated phagocytosis, potassium efflux, and ROS production are the common features

associated with NLRP3 inflammasome activation. In our experiments, β-glucan–induced IL-1β release was abrogated by the inhibitors of ROS formation and potassium efflux (Fig. 7). Whether these signals are either directly recognized by NLRP3 inflammasome or indirectly sensed through cytoplasmic proteins that modulate inflammasome activity remains to be studied. It is probable that low intracellullar potassium acts as an additional requirement for NLPR3 activation by favoring the assembly of NLRP3 inflammasome. ROS production is a highly conserved signal involved in damage and stress sensing and therefore it is reasonable to understand that ROS formation is also involved in inflammasome activation.

Mutations of NLRP3 are associated with several autoinflammatory diseases. In addition, autoinflammatory diseases, such as gout, pseudogout, silicosis, and asbestosis, are associated with enhanced activation of NLRP3 inflammasome (8), and inflammation plays an important role even in atherosclerosis and diabetes. Interestingly, it has been previously shown that β-glucans can trigger autoimmune arthritis in genetically susceptible mice (48) and it is probable that inflammasome activation triggered by β-glucans is involved in this process. Our findings suggest that fungal β-glucans may contribute to inflammatory and autoimmune diseases in humans by activating NLRP3 inflammasome.

In conclusion, the data presented in our study suggest that large (1,3)-β-glucans, including curdlan, GBY, paramylon, and ZYM trigger IL-1β–mediated immune response by providing two separate signals in human primary macrophages. First, the β-glucans are recognized through the membrane bound dectin-1 receptor, which activates the IL-1β mRNA expression and the pro–IL-1β production. Second, the β-glucans are recognized by the cytosolic NLRP3 inflammasome complex resulting in the caspase-1–dependent IL-1β secretion. The β-glucan–induced NLRP3 inflammasome activation is also dependent on dectin-1/Syk signaling pathway. The production of IL-1β precursor and the secretion of the active IL-1β are usually considered to be triggered by two separate substances. The IL-1β transcription is typically activated by PAMPs, and in parallel, DAMPs are required for the activation of IL-1β secretion. In contrast, our results show that (1,3)-β-glucan can perform both PAMP and DAMP functions to trigger IL-1β production in human macrophages. The ability of the major fungal cell wall component to induce the IL-1β production on its own, suggests that the rapid production of IL-1β is an important part of the antifungal defense.

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Disclosures

The authors have no financial conflicts of interest.

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