Recognition and Blocking of Innate Immunity Cells by *Candida albicans* Chitin[▽][†]

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Chitin is a skeletal cell wall polysaccharide of the inner cell wall of fungal pathogens. As yet, little about its role during fungus-host immune cell interactions is known. We show here that ultrapurified chitin from *Candida albicans* cell walls did not stimulate cytokine production directly but blocked the recognition of *C. albicans* by human peripheral blood mononuclear cells (PBMCs) and murine macrophages, leading to significant reductions in cytokine production. Chitin did not affect the induction of cytokines stimulated by bacterial cells or lipopolysaccharide (LPS), indicating that blocking was not due to steric masking of specific receptors. Toll-like receptor 2 (TLR2), TLR4, and Mincle (the macrophage-inducible C-type lectin) were not required for interactions with chitin. Dectin-1 was required for immune blocking but did not bind chitin directly. Cytokine stimulation was significantly reduced upon stimulation of PBMCs with heat-killed chitin-deficient *C. albicans* cells but not with live cells. Therefore, chitin is normally not exposed to cells of the innate immune system but is capable of influencing immune recognition by blocking dectin-1-mediated engagement with fungal cell walls.

Fungal diseases in immunocompetent individuals are mostly restricted to superficial and non-life-threatening infections of the mucosa. However, in patients in which the sentinel activities of the immune system are compromised, fungi can cause systemic disease with mortalities that match, or exceed, those of even the most challenging bacterial septicemias. For example, systemic candidosis due to *Candida albicans* has associated mortality rates of 30 to 40% in the immunocompromised host (21). Recognition of fungal pathogens by innate immune cells is an essential event required for the establishment of host defense mechanisms and therefore protection from mycosis. Identification of microbial molecules, the pathogen-associated molecular patterns (PAMPs) of microbes, is carried out by pattern recognition receptors (PRRs), which are mostly localized in the surface of host immune cells. The *C. albicans* cell wall is the first point of contact with the host tissue and cells, and it contains most of the PAMPs recognized on this fungus (40, 53). The wall is composed of an outer layer of mannoproteins that are heavily glycosylated with N- and O-linked glycans and are attached via glycosylphosphatidylinositol anchors to the inner skeletal layer composed of chitin and β1,6- and

β1,3-glucans (26). Previous studies have established that N- and O-linked glycans are recognized by the mannose receptor (MR) and Toll-like receptor 4 (TLR4), respectively (41, 42, 57), while the glycolipid phospholipomannan (PLM) is recognized by TLR2 (24), and the galectin-3 receptor is involved in the sensing of β1,2-mannose residues (23). β1,3-Glucan, the most abundant sugar polymer in the inner layer of the cell wall, is recognized by dectin-1 and TLR2 (2, 3, 10, 13, 18, 41, 52).

Chitin is a homopolymer of β1,4-*N*-acetylglucosamine (GlcNAc) and is a hallmark component of the cell walls of almost all fungi as well as the cyst walls of pathogenic amoebae, the eggshells and gut linings of parasitic nematodes, and the exoskeletons of invertebrate vectors of human disease, including mosquitoes, sand flies, ticks, and snails. Despite this, little about the role of chitin as a potential PAMP in the recognition of *C. albicans* and other fungal pathogens by immune cells is known (30).

As in *Saccharomyces cerevisiae*, chitin represents 2 to 3% of the *C. albicans* cell wall dry weight under nonstressed conditions (26). However, the chitin content of the wall can increase significantly in response to cell wall damage (54). Despite being a relatively minor component of the *C. albicans* cell wall, chitin is essential for cell shape and viability, forming a layer in the inner walls of yeasts, pseudohyphae, and hyphae, as well as the primary septum of all septa (34). Synthesis of chitin in *C. albicans* is carried out by four chitin synthases (5, 19, 32, 36, 37). Chs1 is essential for *C. albicans* viability and is required for the synthesis of the primary septum and maintaining the integrity of the lateral cell wall (37). *CHS2* encodes a chitin synthase that represents the major measurable activity *in vitro* and which contributes to the synthesis of about 40% of the

chitin present in hyphal cells (19, 36). Chs3 and Chs8 synthesize short- and long-chitin microfibrils, respectively (31). Both enzymes are located at the sites of septum formation in both yeast and hyphal cells (31). In addition, Chs3 is localized at the tip of growing buds and hyphae (31) and synthesizes about 85% of the chitin present in the cell wall of all cell types (5, 35).

Chitin is not present in mammalian cells, and therefore, recognition of chitin by PRRs would represent an appropriate mechanism for detection of fungal invasion. Recent work has revealed several chitin-binding proteins, such as RegIIIg (HIP/PAP), a C-type lectin expressed in the neutrophil-like Paneth cells of the small intestine (6), and FIBCD1, a calcium-dependent acetyl group-binding receptor that is also expressed in the gastrointestinal tract (47), although no chitin receptor in myeloid cells of the immune system has thus far been identified.

Here, we evaluated the role of chitin as a potential PAMP recognized by host innate immune cells. Highly purified, high-molecular-weight chitin was not able to induce pro- or anti-inflammatory cytokine production by human peripheral blood mononuclear cells (PBMCs). However, preincubation of PBMCs with chitin blocked normal recognition of *C. albicans* cells. Accordingly, *C. albicans* cells with high chitin contents stimulated lower-level cytokine responses than cells expressing normal chitin levels at the cell wall, but surprisingly, a *C. albicans chs3Δ* null mutant stimulated less cytokine production by PBMCs than wild-type cells. Experiments with receptor-depleted knockout mouse cells indicated that dectin-1, but not the Mincle (the macrophage-inducible C-type lectin receptor), TLR2, or TLR4, is required for chitin recognition.

MATERIALS AND METHODS

***C. albicans* strains and growth conditions.** *C. albicans* NGY152 (1), CAF2-1 (12), and myco3, a *chs3Δ* null mutant (5), were used in this study. Unless otherwise indicated, cells were grown overnight at 30°C in Sabouraud broth (1% [wt/vol] mycological peptone, 4% [wt/vol] glucose) with shaking at 200 rpm, transferred to fresh medium, and incubated until the yeast cell culture reached mid-log-phase growth (typically 4 h). This ensured consistency in the physiological state of the cells of the inoculum used in these experiments. The cells were collected by centrifugation and washed twice in 20 ml sterile phosphate-buffered saline (PBS), and the concentration was adjusted to 1×10^8 cells/ml. Heat-killed *C. albicans* cells were prepared by incubation at 56°C for 1 h to expose β-glucan on the cell surface. Caspofungin-treated cells were generated by incubating *C. albicans* NGY152 cells in Sabouraud broth supplemented with 0.032 μg/ml caspofungin (Merck Research Laboratories, NJ) for 6 h at 30°C.

Animals and receptor-deficient mouse strains. Experiments were performed with TLR2-deficient (TLR2$^{-/-}$) and TLR4-deficient (TLR4$^{-/-}$) mice (kindly provided by Shizuo Akira, Osaka University, Japan) and wild-type mice (C57BL/6). Dectin-1-deficient (dectin-1$^{-/-}$) mice were produced as previously described (52). Both knockout and wild-type mice were 7 to 9 weeks old, weighing 20 to 25 g, at the time the experiments were performed. The animals were fed standard chow (Hope Farms RMB-H, Woerden, Netherlands) and water *ad libitum*. The day/night cycle was 12 h/12 h. Before use, the animals were allowed to acclimatize for 5 days. The experiments were approved by the Animal Ethics Review Committee of the Radboud University Nijmegen Medical Centre.

Chitin purification and analysis. Chitin is cross-linked covalently to β1,3-glucan and both directly and indirectly to cell wall proteins. Careful and precise purification procedures are required to obtain chitin that is free from other components of the cell wall. Chitin was extracted and purified as described previously (16). Briefly, cells of *C. albicans* NGY152 were grown in YPD broth (1% [wt/vol] yeast extract, 2% [wt/vol] mycological peptone, 2% [wt/vol] glucose) overnight at 30°C with shaking at 200 rpm, washed three times with deionized water, resuspended in 5% (wt/vol) KOH, and boiled for 30 min at 100°C. The preparation was then centrifuged at low speed, and the pellet was washed three times with deionized water, resuspended in 40% H_2O_2-glacial acetic acid (1:1) solution, and autoclaved for 20 min at 121°C. Material was collected by centrifugation, the pellet was washed with water, and the sample was extracted a further

three times in hot 5% KOH with washes between each boiling step. Finally, the chitin was washed repeatedly, resuspended in PBS, and stored at −20°C until use.

To determine the chitin yield in the preparations, samples were hydrolyzed for 3 h with 2 M trifluoroacetic acid at 100°C. The acid was removed by evaporation at 65°C, and the debris was resuspended in deionized water, which was evaporated off, and finally resuspended in deionized water. Samples of 20 μl were analyzed by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) in a carbohydrate analyzer system from Dionex (Surrey, United Kingdom) as described previously (44). High-purity monosaccharide standards used to generate pulsed amperometric response curves were from Sigma-Aldrich (Dorset, United Kingdom). The deacetylation degree of chitin samples was determined by binding to Cibacron brilliant red 3B-A dye (38).

The protein content was determined by the Coomassie protein assay (Perbio Science, Northumberland, United Kingdom). Prior to use, all samples were checked to ensure that they were free of microbial contamination and lipopolysaccharide (LPS). Possible fungal contamination was assessed by incubation of 20-μl samples in YPD broth for 72 h at 30°C. Bacterial contamination was evaluated by both LPS quantification (*Limulus* amebocyte lysate assay; Hycult Biotech) and incubation of samples at 37°C for 72 h in LB broth (1% [wt/vol] tryptone, 0.5% [wt/vol] yeast extract, 0.5% [wt/vol] NaCl). These various controls demonstrated that the chitin purified by the protocol described above was free of glucose and mannose-based polysaccharides and from any microbiological contamination. For a comparison, chitin obtained from a commercial source (Sigma purified powder and BioReagent quality chitin from shrimp shells [catalog no. C9752]) was also acid hydrolyzed and analyzed by HPAEC-PAD.

Cell wall composition. Cell walls were prepared as previously described (33). For β-glucan, mannan, and chitin quantification, cell walls were acid hydrolyzed as described above, and glucose (Glc), mannose (Man), and GlcNAc were quantified by HPAEC-PAD.

Fluorescence microscopy. Cells were grown overnight in Sabouraud broth, and 600-μl samples were inoculated in 50 ml fresh Sabouraud broth and further incubated for 4 h at 30°C. Cells were harvested by low-speed centrifugation, washed twice with sterile PBS, stained with either 25 μg/ml calcofluor white (CFW) or 100 μg/ml fluorescein isothiocyanate-conjugated wheat germ agglutinin (WGA-FITC) and examined by phase differential interference contrast (DIC) and fluorescence microscopy using a Zeiss Axioplan 2 microscope. Images were taken by means of the Openlab system (version 4.04; Improvision, Coventry, United Kingdom) and a Hamamatsu C4742-95 digital camera (Hamamatsu Photonics, Hamamatsu, Japan).

Cytokine stimulation in human PBMCs. Human PBMCs were isolated as previously described (11). Samples of blood from healthy volunteers were analyzed anonymously. PBMCs (5×10^5 cells in 100 μl) from six individual donors were incubated in round-bottom 96-well Nunclon plates (Nunc, Roskilde, Denmark) with 100 μl of chitin samples (different concentrations ranging from 5 to 200 μg/ml), heat-killed *Salmonella enterica* serovar Typhimurium cells, 1 μg/ml Pam₃CSK₄, 10 ng/ml LPS from *Escherichia coli*, and the live or heat-killed *C. albicans* strains at a concentration of 1×10^5 yeast cells/ml, unless otherwise indicated. After 24 h of incubation at 37°C under 5% (vol/vol) CO_2, the preparations were centrifuged and supernatants collected and stored at −70°C until assayed. For interleukin-1α (IL-1α) quantification, stimulated PBMCs were disrupted by three sequential freeze (−80°C) cycles, and cytokine concentrations in the homogenates were quantified. All the cytokines levels were analyzed by enzyme-linked immunosorbent assays (ELISAs) using commercial kits from R&D Systems (Abingdon, United Kingdom). Unless otherwise indicated, for the blocking assays, PBMCs were preincubated for 60 min at 37°C with 10 μg/ml chitin before stimulation.

Cytokine production by murine peritoneal macrophages. Groups of five knockout mice and their control littermates were sacrificed. Resident peritoneal macrophages were harvested by injection of 4 ml of sterile PBS containing 0.38% sodium citrate. After being washed, the cells were resuspended in RPMI 1640 containing 1 mM pyruvate, 2 mM L-glutamine, 100 μg/ml gentamicin, and 2% fresh mouse plasma (culture medium). Cells were cultured in 96-well microdilution plates (Greiner, Alphen, Netherlands) at 10^5 cells per well, in a final volume of 200 μl. The cells were stimulated with either control medium or chitin (final concentration, 200 μg/ml). After incubation for 24 h at 37°C, the plates were centrifuged ($500 \times g$ for 10 min) and the supernatant was collected and stored at −80°C until cytokine assays were performed.

Receptor binding experiments. Surface plasmon resonance experiments were performed with a Biacore T100 machine (GE Healthcare, Uppsala, Sweden) as previously described (43). Streptavidin (Sigma, Gillingham, United Kingdom) was covalently coupled to carboxymethylated dextran matrix CM5 research-

grade chips via primary amines with immobilization levels of typically about 3,000 response units (RU). The biotinylated extracellular domain of Mincle was immobilized at the required levels (200 to 2,000 RU for equilibrium-based experiments) by injection over the streptavidin-coupled surfaces. The signal from flow cells coated with Mincle was compared to the signal from mock-coupled cells or cells coupled with an irrelevant protein to control for any nonspecific effects. For experiments in the reverse orientation, chitin was tethered to the chip surface by aldehyde coupling and Mincle was injected over the biosensor surface. Chitin concentrations used in injections ranged from 1 μg/ml up to 1 mg/ml.

Immuno MaxiSorp 96-well ELISA plates (Nunc) were coated with chitin or bovine serum albumin (BSA) (Sigma) at the indicated concentrations overnight at 4°C. Wells were blocked with 1% BSA in 25 mM HEPES, pH 7.4, 150 mM NaCl, and 0.05% Tween 20 and then incubated with 0.5 μg biotinylated Mincle or biotinylated irrelevant protein (NKG2D) for 2 h at room temperature. Wells were washed with 25 mM HEPES, pH 7.4, 150 mM NaCl, and 0.05% Tween 20, and detection was carried out with streptavidin peroxidase (Sigma) and tetramethylbenzidine (TMB) (Sigma).

Fluorescence-activated cell sorter (FACS) analysis of the β-glucan and chitin particles by using a soluble Fc–dectin-1 chimeric receptor was performed as described previously (20).

Statistical analyses. Experiments with human PBMCs were conducted in duplicate with a total of 6 healthy donors. Assays involving murine peritoneal macrophages were carried out in duplicate with a total of 9 mice of each strain or genetic background. The Mann-Whitney U test was applied to analyze the data obtained in this work, with a level of significance between groups set as a P value of <0.05. The data are given as means ± standard deviations (SDs).

RESULTS

Chitin does not stimulate cytokine production but blocks the recognition of *C. albicans*. We investigated whether chitin was recognized as a PAMP by the PRRs of human PBMCs. We tested two batches of commercially available chitin preparations and found that these contained contaminants that could potentially mislead interpretation of experiments. Hydrolysates from purified chitin obtained from a commercial source contained trace amounts of glucose and protein and several other unidentified contaminants (Fig. 1C). Therefore, we purified chitin from *C. albicans* cell walls and rigorously tested this chitin for chemical and microbiological purity. Chitin isolated following the chemical extraction described in Materials and Methods has been demonstrated to be ultrapure (15–17), and as expected, our chitin preparations were protein, mannan, and glucan free, as tested by HPAEC-PAD and protein quantification (Fig. 1B). This ultrapure chitin was also LPS free (data not shown) and negative for microbiological culture. The deacetylation degree of the chitin preparations was determined to be 5%.

Ultrapurified chitin was not able to induce cytokine production from PBMCs, even when used at concentrations up to 200 μg/ml (data not shown). However, pretreatments of human PBMCs with chitin blocked the recognition of *C. albicans*, as shown by the reduction in the levels of induced cytokines. Preincubation of PBMCs with 10 μg/ml chitin led to a significant reduction in the levels of tumor necrosis factor alpha (TNF-α), IL-6, and IL-1β stimulated by live *C. albicans* cells (Fig. 2A), while no changes were observed in the stimulated levels of IL-10 and gamma interferon (IFN-γ). When a similar experiment was conducted with heat-killed *C. albicans* yeast cells, the same trends in TNF-α, IL-6, and IL-1β production were observed (Fig. 2B). However, blocking with chitin abolished the IFN-γ stimulation by heat-killed *C. albicans* cells. Different from the results obtained with live cells, the stimulation of the anti-inflammatory cytokine IL-10 was significantly
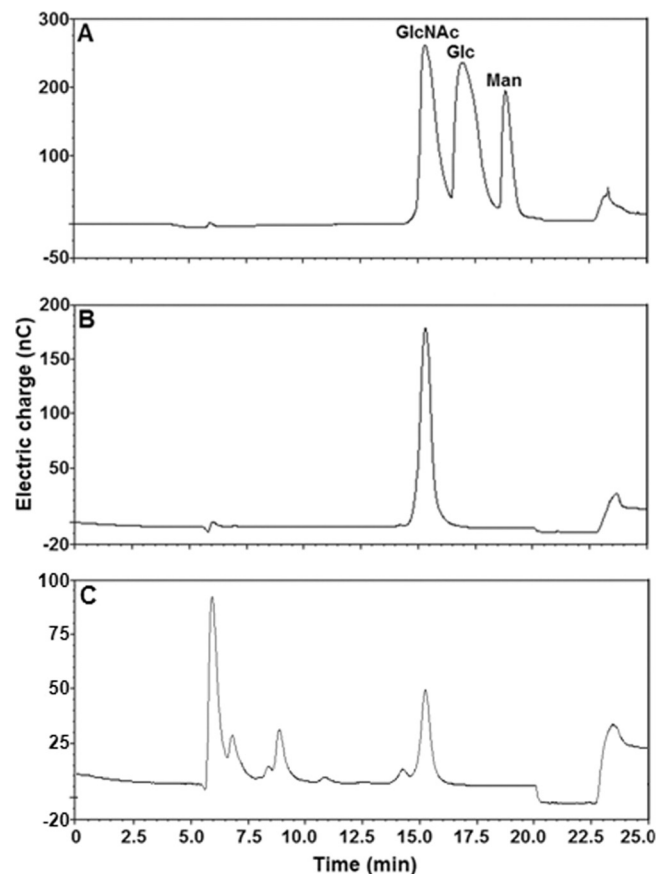

FIG. 1. Characterization of the chitin isolated from *C. albicans*. Chitin preparations were isolated and acid hydrolyzed as described in Materials and Methods. Samples containing 20-μl aliquots of hydrolyzed sample from *C. albicans* (panel B) or from shrimp shells (Sigma; panel C) were analyzed by HPAEC-PAD using a CarboPac PA200 analytical column. For panel A, high-purity standards (1 μg each; Sigma) were mixed and separated by HPAEC-PAD as indicated. GlcNAc, *N*-acetylglucosamine; Glc, glucose; Man, mannose.

reduced after 1 h of preincubation with chitin (Fig. 2B). Similar results were obtained when heat-killed *S. cerevisiae* cells were used to stimulate cytokine production or when ultrapure chitin from this organism was used to treat PBMCs before challenge with *C. albicans* (data not shown).

To test whether chitin-mediated blocking of immune recognition was a steric effect due to the masking of receptors on monocyte cell surfaces, we tested whether chitin-treated monocytes remained responsive to bacterial LPS. Heat-killed *Salmonella* Typhimurium cells still stimulated cytokine production from monocytes that had been exposed to chitin for 1 h, indicating that LPS-mediated recognition still occurred in the presence of chitin (Fig. 2C). These data therefore suggest that the blocking effect of chitin may be due a ligand-receptor interaction rather than unspecific binding of the polysaccharide to the cell surface of the monocytes.

***C. albicans* cells with high chitin content stimulate reduced cytokine production.** Since chitin had a blocking effect on cytokine production stimulated by *C. albicans*, we next compared the levels of cytokine production stimulated by wild-type control and caspofungin-treated yeast cells. We have previously
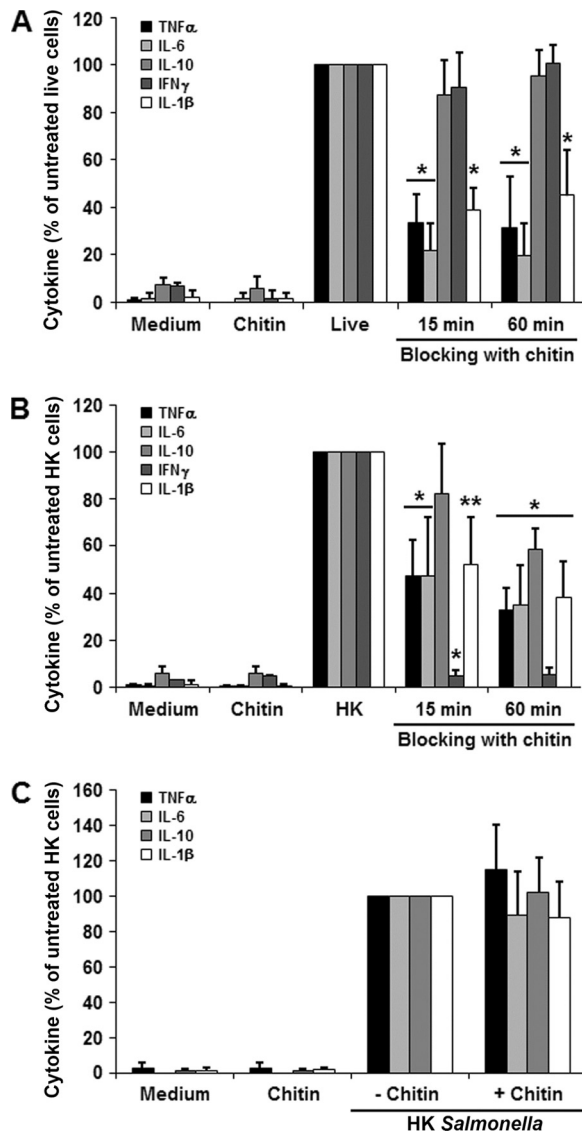
FIG. 2. Chitin blocks the recognition of heat-killed and live *C. albicans*. Human PBMCs were preincubated at 37°C for the indicated times with 10 μg/ml chitin and then stimulated with 1×10^5 yeasts/ml of either live (panel A) or heat-killed (HK; panel B) *C. albicans* NGY152. For controls, PBMCs were also stimulated with medium or 10 μg/ml chitin. TNF-α, IL-6, IL-10, IFN-γ, and IL-1β were measured after 24 h of incubation. A similar experiment was conducted with *S.* Typhimurium as the stimulus (panel C). The results are pooled duplicate data from three separate experiments with a total of 6 volunteers (means ± SDs). * and **, *P* values of <0.01 and <0.05, respectively, for results obtained with chitin-treated cells compared to those obtained with untreated cells.
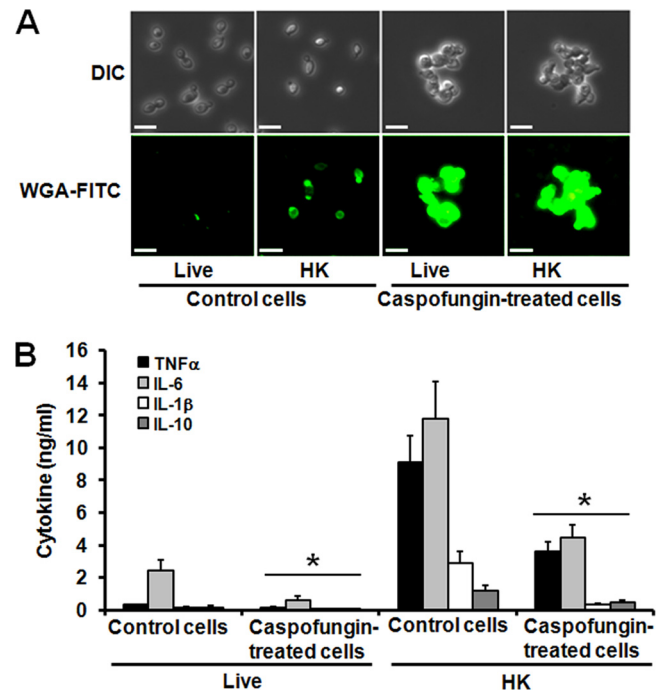

FIG. 3. Caspofungin-treated *C. albicans* cells have high chitin contents at the cell wall (A) and stimulate reduced levels of cytokines (B). *C. albicans* NGY152 was grown for 6 h in Sabouraud medium supplemented with 0.032 μg/ml caspofungin and was stained with the fluorescent lectin WGA-FITC (panel A) or used to stimulate cytokine production by human PBMCs (panel B). For panel A, all pictures were taken at the same exposure time, and WGA-FITC is able to bind the accessible chitin only at the cell surface. The scale bars represent 10 μm. In panel B, the results are pooled duplicate data from three separate experiments with a total of 6 volunteers (means ± SDs). *, *P* value of <0.05 for results obtained with caspofungin-treated cells compared to those obtained with control cells. IL-1β concentrations stimulated by live control cells and live caspofungin-treated cells were 0.17 ± 0.01 ng/ml and 0.06 ± 0.03 ng/ml, respectively, and the IL-10 concentrations under these conditions were 0.17 ± 0.08 ng/ml and 0.05 ± 0.01 ng/ml, respectively. Control cells were untreated *C. albicans* NGY152. HK, heat-treated *C. albicans* cells.

reported that incubation of *C. albicans* cells with sublethal concentrations of caspofungin leads to increased levels of chitin at the cell wall, as a protective mechanism against the drug effect (54). *C. albicans* NGY152 yeast cells growing in the presence of 0.032 μg/ml caspofungin had a 3-fold increase in cell wall chitin content compared with that of untreated cells (82 ± 8 and 26 ± 4 μg chitin/mg of cell wall dry weight, respectively; *P* < 0.002), but no significant changes were recorded for the cell wall mannan (325 ± 20 and 304 ± 12 μg

mannan/mg of cell wall dry weight with and without caspofungin, respectively) or β-glucan content (521 ± 19 and 541 ± 15 μg glucan/mg of cell wall dry weight with and without caspofungin, respectively). Heat killing of *C. albicans* cells exposes β-glucan at the cell wall surface (14, 18, 56). In order to determine whether heat killing similarly results in chitin exposition on the cell wall surface, live and heat-killed *C. albicans* cells were stained with the chitin-binding lectin WGA-FITC and the fluorochrome CFW, which have specificity for GlcNAc residues and chitin (39). It was reasoned that small fluorochromes such as CFW would permeate intact live yeast cells and bind chitin, while large molecules like WGA would not (46). As expected, live *C. albicans* cells were barely stained with WGA-FITC, whereas a clear binding of this lectin to chitin was observed with heat-killed cells (Fig. 3A). In accordance with the cell wall composition results, live and heat-killed caspofungin-treated cells bound more WGA-FITC than untreated cells, with a stronger fluorescence signal (Fig. 3A). Therefore, in live cells with intact cell walls, most of the chitin appears to be hidden in the cell wall inner layer, but in caspofungin-treated
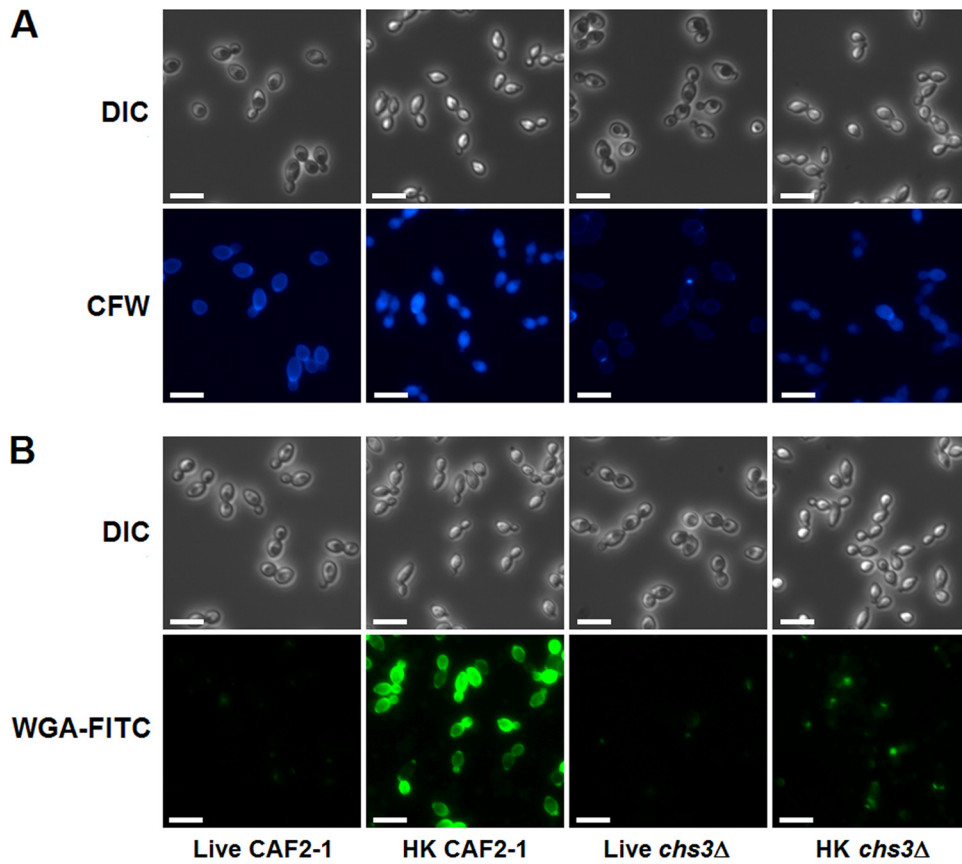
FIG. 4. Staining of chitin in *C. albicans* wild-type (CAF2-1) and *chs3*Δ null mutant cells by using calcofluor white (A) and the fluorescent lectin WGA-FITC (B). The former stain detects chitin in all cells, while WGA is able to bind to accessible chitin only at the cell surface. The scale bars represent 10 μm. HK, heat-treated *C. albicans* cells.

C. albicans cells, this polysaccharide is present in both the inner and external cell wall layers.

Next, we compared the cytokine profiles stimulated by live and heat-killed control and caspofungin-treated cells. As reported previously (18), heat-killed wild-type cells stimulated a greater cytokine response than live cells (Fig. 3B). The TNF-α, IL-6, IL-1β, and IL-10 levels stimulated by either live or heat-killed caspofungin-treated cells were significantly lower than the cytokine production levels stimulated with untreated cells (Fig. 3B). Therefore, these results indicated that cells with high chitin content at the cell wall stimulate a reduced level of cytokine production.

Differential cytokine stimulation by the *C. albicans* *chs3*Δ null mutant. Chitin is an inner cell wall component of *C. albicans* that represents approximately 2 to 3% of the cell wall dry weight. We compared levels of cytokine stimulation by yeast cells of wild-type control and *C. albicans* *chs3*Δ null mutant cells (5). The *chs3*Δ null mutant has an 80 to 90% reduction in cell wall chitin content. We confirmed that the *chs3*Δ null mutant had a significantly lower cell wall chitin content than the parental strain (3.0 ± 1.0 and 22.6 ± 3 μg chitin/mg of cell wall dry weight, respectively [$P < 0.005$]) and had a slightly increased β-glucan content (637 ± 17 versus 528 ± 14 μg glucose/mg of cell wall dry weight [$P < 0.005$]) and reduced mannan levels (214 ± 11 versus 296 ± 18 μg mannose/mg of cell wall dry weight [$P < 0.005$]). CFW stained live

and heat-killed wild-type control cells equally well (Fig. 4A), but little staining of live cells with WGA-FITC was observed (Fig. 4B), whereas heat-killed cells were clearly stained with WGA-FITC (Fig. 4B). Confirming its reduced chitin content, the *chs3*Δ null mutant stained less with both WGA-FITC and CFW than wild-type cells (Fig. 4).

When we compared the levels of cytokine stimulation by wild-type and chitin-deficient cells, levels of TNF-α, IL-6, and IFN-γ production by live or heat-killed *chs3*Δ null mutant cells were similar to those displayed by wild-type cells (Fig. 5A, B, and F). However, levels of stimulation of IL-1α, IL-1β, and IL-10 were significantly reduced upon stimulation with heat-killed *chs3*Δ mutants but not with live *chs3*Δ cells (Fig. 5C to E). Therefore, differences in the stimulation of IL-1α, IL-1β and IL-10 from human PBMCs by the *chs3*Δ mutant was observed only in cells that had the inner cell wall layer exposed by heat killing. This suggests that the chitin in the cell wall directly influences immune recognition, presumably by interacting with a specific receptor on the cell membrane of PBMCs.

Dectin-1, but not Mincle, TLR2, or TLR4, is required for chitin recognition. To investigate the identity of the receptor involved in the chitin recognition, we assessed whether chitin could block cytokine production triggered by specific PAMP-PRR interactions. We first assessed whether chitin could block cytokine production stimulated by Pam_3CSK_4- and bacterial LPS-specific ligands for TLR2 and TLR4, respectively. While
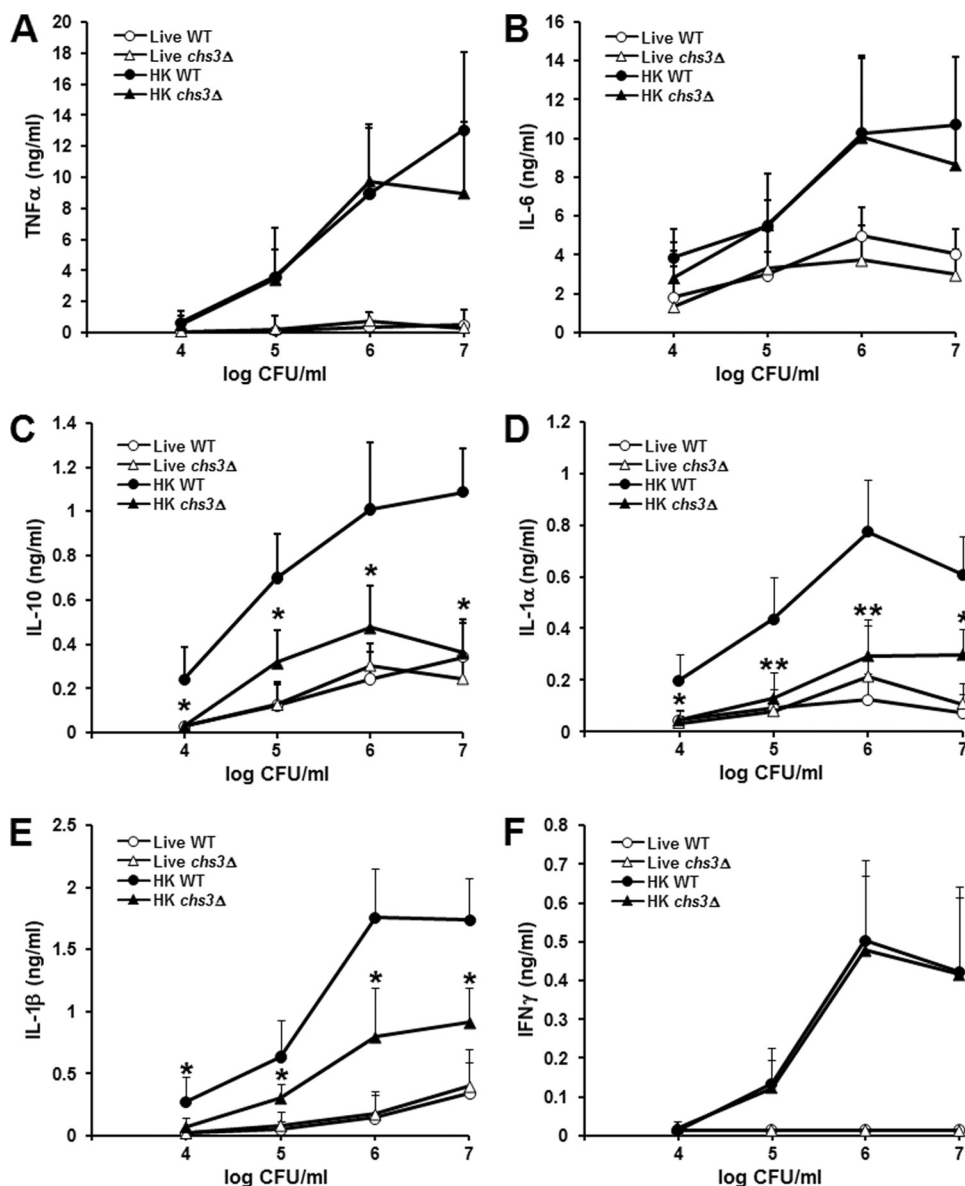
FIG. 5. Cytokine production in human PBMCs stimulated by live or heat-killed (HK) yeast cells of a *C. albicans* wild-type strain (CAF2-1) (WT) and a *chs3Δ* null mutant. After 24 h of incubation, supernatants were removed and used to measure the levels of TNF-α (panel A), IL-6 (panel B), IL-10 (panel C), IL-1α (panel D), IL-1β (panel E), and IFN-γ (panel F). The results are pooled duplicate data from three separate experiments with a total of 6 volunteers (means ± SDs). * and **, *P* values of <0.05 and <0.01, respectively, for results obtained with *chs3Δ* null mutant cells compared to those obtained with wild-type cells.

chitin blocked the recognition of live *C. albicans* yeast cells by PBMCs, leading to significant reduction in the stimulation of TNF-α, IL-6, and IL-1β but not IL-10 (Fig. 6), preincubation of PBMCs with chitin did not block the stimulation of TNF-α, IL-6, IL-1β, or IL-10 by LPS and Pam₃CSK₄ (Fig. 6A to D). This suggests that neither TLR2 nor TLR4 is involved in the recognition of chitin. In order to confirm this observation, assays using chitin as a blocking agent were carried out with peritoneal macrophages from wild-type, TLR2⁻/⁻, and TLR4⁻/⁻ mice. As was already seen with human PBMCs, chitin blocked the recognition of *C. albicans* by mouse peritoneal macrophages, and this effect was independent of the presence of TLR2 or TLR4 receptors, as indicated by the signifi-

cant reduction in TNF-α, IL-6, IL-1α, and IL-1β (Fig. 7A to D) produced in the presence of chitin. Thus, these data confirmed that neither TLR2 nor TLR4 is involved in chitin recognition.

We also demonstrated that the Mincle receptor does not seem to bind chitin directly, as surface plasmon resonance and ELISAs showed that this receptor does not bind this oligosaccharide (data not shown). Next, the role of dectin-1 in the recognition of this cell wall polymer was investigated. By FACS analysis, we showed that dectin-1 did not bind chitin directly (Fig. 8A); however, chitin did not block the cytokine production stimulated by *C. albicans* cells in peritoneal macrophages from dectin-1-knockout mice (Fig. 8B). Therefore, dectin-1 does not bind chitin but is required for the recognition of chitin
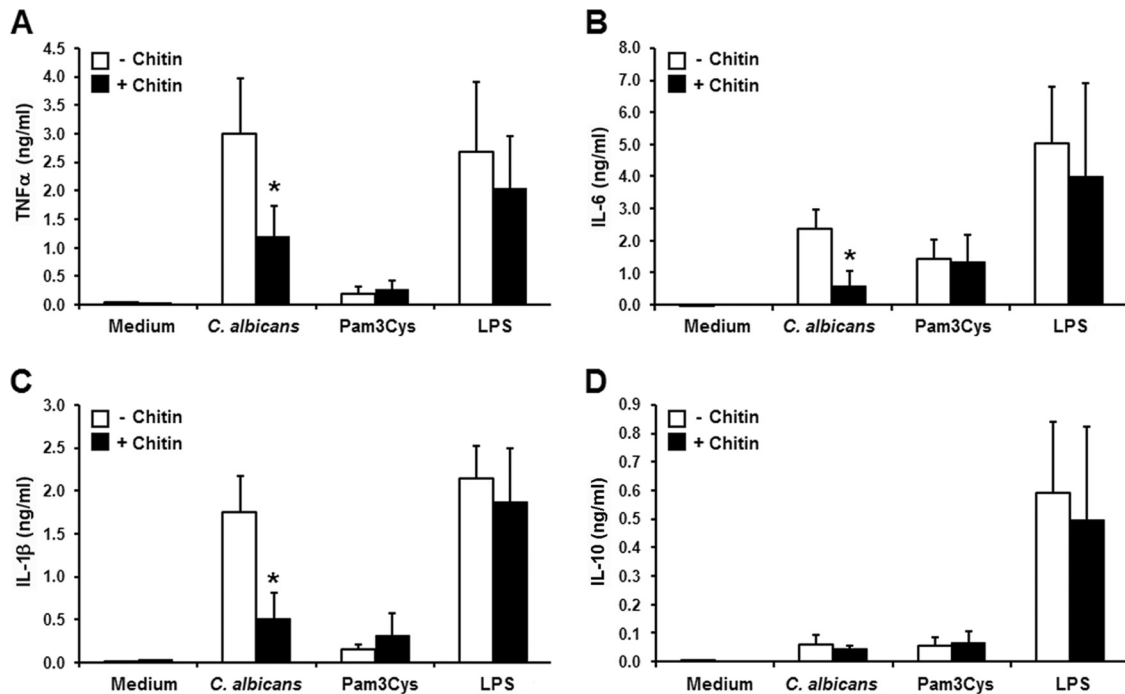
FIG. 6. Chitin does not block the recognition of specific agonists of TLR2 and TLR4. Human PBMCs were preincubated with 10 μg/ml chitin for 60 min and then stimulated with live *C. albicans* NGY152, 1 μg/ml Pam$_3$CSK$_4$, or 10 ng/ml LPS from *E. coli*. After 24 h of incubation, supernatants were assayed for TNF-α (panel A), IL-6 (panel B), IL-1β (panel C), and IL-10 (panel D). The results are pooled duplicate data from two separate experiments with a total of 8 volunteers (means ± SDs). *, *P* value of <0.01 for results obtained with chitin-treated cells compared to those obtained with untreated cells.

and for the ability of this cell wall component to block immune recognition of *C. albicans*.

DISCUSSION

To date, little attention has been paid to the role of chitin in the immune recognition of fungal cells. Recent studies have demonstrated that β1,3-glucan in the cell wall can become exposed either by treatment with caspofungin, which inhibits β1,3-glucan synthesis, or by heat treatment, which leads to perturbation or damage of the normal architecture of the cell wall (18, 22, 27, 56). In addition, yeast bud scars are a site where β1,3-glucan and chitin are naturally exposed and therefore available for recognition by immune cells (14). Inner cell wall layers can therefore participate in the process of recognition of fungal pathogens by the innate immune system (40). Chitin has also been shown to influence the adhesion of *C. albicans* to a range of host cells and substrates, suggesting that some chitin is naturally exposed at the fungal cell surface (25, 29, 48). Therefore, there is no *prima face* reason why chitin should not also play a role in the recognition of fungal pathogens by the innate immune system. Indeed, recent studies have shown that nematode chitin plays a role in the activation of eosinophils (45), and chitin engagement with a range of immune cell receptors has been reported (49–51).

In this study, we demonstrated that chitin is involved in the recognition of *C. albicans* by mononuclear cells. Although ultrapurified chitin apparently did not stimulate cytokine production directly, it was able to block *C. albicans* recognition and cytokine production by PBMCs. Chitin did not affect cy-

tokine induction by other stimuli, such as *S.* Typhimurium cells, Pam$_3$CSK$_4$, or LPS, indicating that the blocking effect was not due to steric masking or complexing of all receptors by nonspecific binding of the polymer at the surface of the immune cells.

Here, we found that ultrapurified fungal chitin does not directly stimulate production of cytokines from PBMCs. This contrasts with previous reports indicating that chitin particles are able to stimulate IL-12, IL-10, TNF-α, and IFN-γ production from a range of cell types (7, 50, 51). In these reports, it was demonstrated that this response was obtained only when 1- to 10-μm chitin particles, which were small enough to be phagocytosed, were used to stimulate the immune cells (7, 50). In addition, 40- to 70-μm chitin fragments, but not larger ones, are able to stimulate TNF-α production through a phagocytosis-independent mechanism (7). The *C. albicans* cell wall contains both long and short chitin chains (15, 16, 31), and it is not yet known whether chitin fibrils of different architecture interact differently with receptors on immune cells. A precedent for this possibility is evident in the observation that 40- to 70-μm chitin fragments were recognized via TLR2 and dectin-1 but phagocytable chitin particles required additional recognition through mannose receptors (7). In addition, some isoforms of β1,3-glucan, such as laminarin and glucan-phosphate, are blockers of the immune response, while β1,3-glucan in zymosan elicits a strong proinflammatory response in myeloid cells via interactions with dectin-1 (4, 18, 33).

We found that chitin was able to block the recognition of *C. albicans* yeast cells, indicating that this cell wall component is
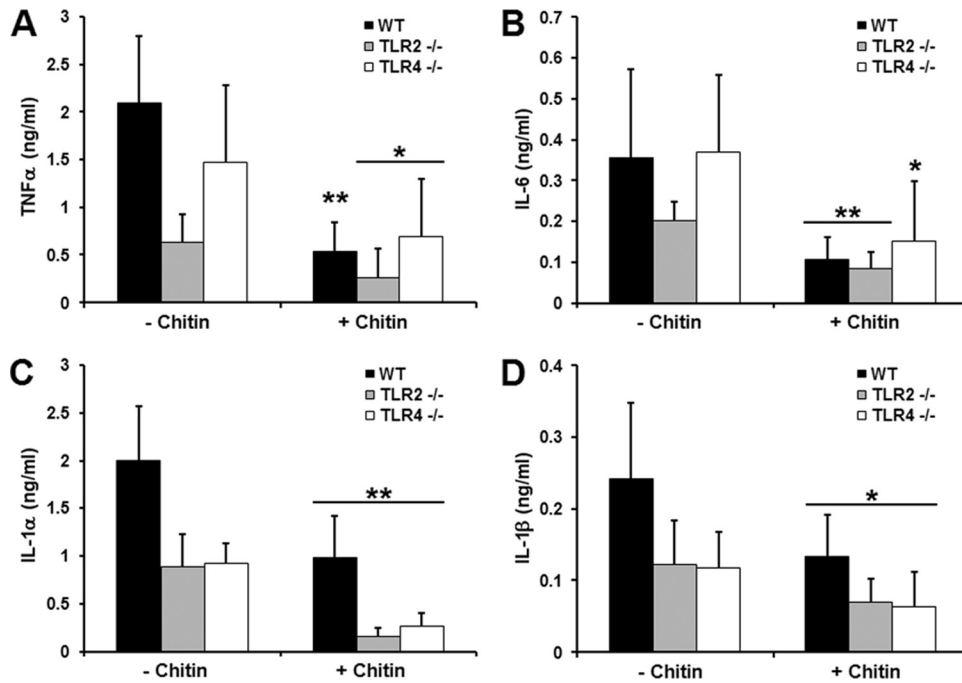
FIG. 7. Blocking effect of chitin on the *C. albicans* recognition is not TLR2 or TLR4 dependent. Peritoneal macrophages from C57BL/6J (WT), TLR2$^{-/-}$, and TLR4$^{-/-}$ mice were preincubated at 37°C for 60 min with 10 μg/ml chitin and then stimulated with heat-killed *C. albicans* NGY152 at a concentration of 1×10^5 yeasts/ml. After 24 h of incubation, supernatants were removed and assayed for TNF-α (panel A), IL-6 (panel B), IL-1α (panel C), and IL-1β (panel D). The results are pooled duplicated data from two separate experiments with a total of 9 animals per group (means ± SDs). * and **, P values of <0.05 and <0.01, respectively, for results obtained with chitin-treated cells compared to those obtained with untreated cells.

recognized at the immune cell surface and that this ligand-receptor interaction affects the normal recognition of *C. albicans*. This observation was confirmed by the observed reduction in cytokine production stimulated by caspofungin-treated cells, which had significantly more chitin at the cell wall but normal β-glucan and mannan contents. In addition, a *chs3*Δ null mutant showed alterations in the ability to stimulate cytokine production. This mutant had significantly less chitin in its cell wall and a slight reduction (28%) in the mannan content. Mannans play a major role in the recognition of live *C. albicans* (41), and since recognition of live *chs3*Δ cells was not

greatly altered and elicited cytokine stimulation similar to that elicited by the wild-type cells, it is unlikely that the change in cell wall mannan levels could affect the recognition of the *chs3*Δ null mutant by PBMCs. However, heat killing of *C. albicans* was shown to reveal chitin at the cell surface, and heat-killed *chs3*Δ yeast cells stimulated less cytokine production than heat-killed wild-type cells, despite the fact that they had more β-glucan in the cell wall. The surprising result that some cytokines were reduced rather than increased in the *chs3*Δ null mutant suggests that chitin may also act as a scaffold or anchor for cell wall molecules that are positive activators
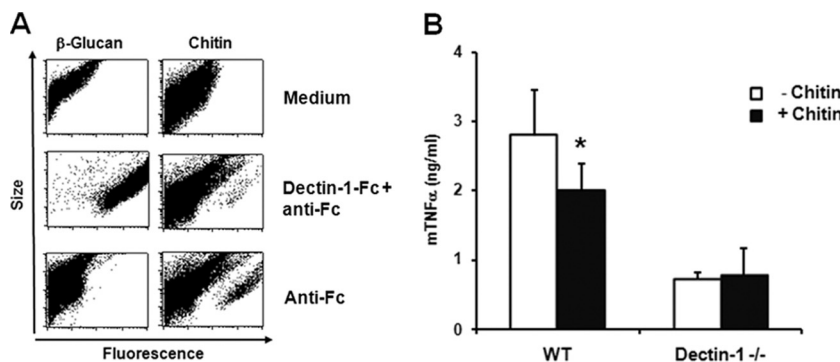

FIG. 8. Dectin-1 is required for the blocking effect of chitin on *C. albicans* recognition. (A) FACS analysis of chitin or β-glucan preparations incubated as described in the text. (B) Peritoneal macrophages from C57BL/6J (WT) and dectin-1$^{-/-}$ mouse PBMCs were preincubated at 37°C for 60 min with 10 μg/ml chitin and then stimulated with heat-killed *C. albicans* NGY152 at a concentration of 1×10^5 yeasts/ml. After 24 h of incubation, supernatants were removed and assayed for TNF-α production. The results are pooled duplicated data from a total of 9 animals per group (means ± SDs). *, a P value of <0.05 for results obtained with chitin-treated cells compared to those obtained with untreated cells.

during immune recognition (e.g., a subfraction of mannoproteins that exist only in the deep cell wall that could stimulate IL-1α, IL-1β, and IL-10). Alternatively, chitin may induce a different response as a pure molecule than in the context of a complex, intact cell wall. Nevertheless, this data set underlines the potential importance of chitin for *C. albicans* recognition. β1,3-Glucan is recognized by a dectin-1—TLR2 receptor complex, and this interaction along with mannose receptor–*N*-mannan accounts for most of the cytokine production when heat-treated *C. albicans* cells are used to stimulate cytokine production (18).

The levels of TNF-α, IL-6, and IFN-γ production were unaffected by reduction of the chitin content in the *chs3Δ* null mutant, which may suggest that different adaptor molecules participate in eliciting cytokine responses triggered by this polymer. A similar observation has been made for the recognition of β1,3-glucan by dectin-1 and TLR2. While dectin-1 uses Syk kinase and the adaptor CARD9 to stimulate IL-10, TLR2 via the adaptor MyD88 is required for the production of IL-12p40 (9, 40), while both pathways collaborate in TNF-γ stimulation (10).

TLR4 recognizes O-linked mannan, while TLR2 participates in β-glucan recognition. Our data suggest that neither of these receptors is involved in the recognition of chitin but contrast with recently reported studies (7, 8) in which chitin particles of small size were able to induce stimulation of IL-17, IL-12, IL-23, IL-10, and TNF-α in macrophages via a MyD88- and TLR2-dependent pathway. The reasons for these differences are not yet clear, but they presumably reflect differences in the cell types used or possibly in the purity of chitin preparations. It is unlikely that the extraction and purification protocols used in this work may affect the native structure of cell wall chitin, since similar methodologies have been used to characterize the structure and architecture of chitin molecules, and it has been demonstrated that the morphology of chitin is variable in different fungi despite the use of the same extraction method (15, 31).

It has been reported that the C-type lectin NKR-P1 receptor is able to recognize chito-oligomers and that this interaction leads to the activation of natural killer cells in rats (49). Studies using soluble mannan as a blocking agent have suggested that the mannose receptor of murine spleen cells is involved in the recognition of chitin (51); more recently, TLR2 and the MyD88 adaptor have been proposed to be involved in the recognition of this cell wall polymer (8). Here, we demonstrated that Mincle, a macrophage-inducible C-type lectin involved in the immune recognition of *C. albicans* (55), does not seem to bind chitin directly. Evidence indicated that although dectin-1 is not the receptor for chitin, it is required for the blocking effect of chitin on the recognition of *C. albicans*. Accordingly, it has been recently reported that chitin is able to induce IL-10 production through a dectin-1- and Syk-dependent signaling pathway (7, 28). Our data therefore indicate that chitin recognition is a complex process and is likely to involve collaboration between dectin-1 and another, yet-to-be-described PRR.

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