Macrophage Internalization of Fungal β -Glucans Is Not Necessary for Initiation of Related Inflammatory Responses

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Cell wall β -glucans are highly conserved structural components of fungi that potently trigger inflammatory responses in an infected host. Identification of molecular mechanisms responsible for internalization and signaling of fungal β -glucans should enhance our understanding of innate immune responses to fungi. In this study, we demonstrated that internalization of fungal β -glucan particles requires actin polymerization but not participation of components of caveolar uptake mechanisms. Using fluorescence microscopy, we observed that uptake of 5-([4,6-dichlorotriazin-2-yl] amino)-fluorescein hydrochloride-Celite complex-labeled *Saccharomyces cerevisiae* β -glucan by RAW macrophages was substantially reduced in the presence of cytochalasin D, which antagonizes actin-mediated internalization pathways, but not by treatment with nystatin, which blocks caveolar uptake. Interestingly, β -glucan-induced NF- κ B translocation, which is necessary for inflammatory activation, and tumor necrosis factor alpha production were both normal in the presence of cytochalasin D, despite defective internalization of β -glucan particles following actin disruption. Dectin-1, a major β -glucan receptor on macrophages, colocalized to phagocytic cups on macrophages and exhibited tyrosine phosphorylation after challenge with β -glucan particles. Dectin-1 localization and other membrane markers were not affected by treatment with cytochalasin D. Furthermore, dectin-1 receptors rather than Toll-like receptor 2 receptors were shown to be necessary for both efficient internalization of β -glucan particles and cytokine release in response to the fungal cell wall component.

Most pathogenic fungi possess a β -glucan-rich cell wall comprised of glucose residues arranged in β -(1,3)-D-glucopyranosyl polymers with associated β -(1,6)-D-glucopyranosyl side chains having various length and frequency distributions (5, 13, 20). Fungal β -glucans possess many of the characteristics attributed to pathogen-associated molecular pattern molecules (PAMPs). β -Glucans are highly conserved structural components of the fungal cell wall that potently trigger innate immune responses. Previous studies have demonstrated that β -glucans stimulate the release of inflammatory mediators, including tumor necrosis factor alpha (TNF- α), interleukin-1, MIP-2, eicosanoids, and reactive oxidants (9, 24, 25). Moreover, we have previously demonstrated that β -glucans from the opportunistic pathogen *Pneumocystis carinii*, as well as *Saccharomyces cerevisiae*, act as potent inducers of macrophage activation through NF- κ B translocation utilizing cellular receptors and signaling pathways distinct from those that mediate cellular activation in response to lipopolysaccharide (LPS) (14).

Macrophages are professional phagocytes that are one of the first lines of defense provided by the innate immune system. Recognition and internalization of particles are complex processes involving unique mechanisms and subsequent responses dictated by the nature of the ingested particle (1). Macrophages use a variety of mechanisms for internalization, including pinocytosis, receptor-mediated endocytosis, caveola-mediated uptake mechanisms, and phagocytosis. The first two pathways are usually independent of actin polymerization; in-

stead, they utilize clathrin-based mechanisms and are generally involved in uptake of small molecular ligands. Caveolar uptake mechanisms have been implicated in the internalization of viruses and some bacteria (10, 11, 15). In contrast, phagocytosis, the uptake of particulate material, occurs through actin-dependent mechanisms. However, the internalization of particles is a heterogeneous event which involves molecules that are not exclusive for one particular mechanism but may participate in other processes within the cell.

Fungal cell wall β -glucans have been shown to interact with several receptors on the surface of macrophages (2, 19, 22). Recently, dectin-1 has been the focus of intense investigation. Dectin-1, a small type II receptor expressed on the cell surface of innate immune cells, including macrophages and dendritic cells, has been shown to mediate the response of macrophages to intact yeast and zymosan (2). However, the exact mechanisms of receptor binding and internalization of purified β -glucan particles, as well as downstream signaling pathways leading to macrophage activation and proinflammatory signaling, have not been fully studied yet.

Although considerable efforts have been directed toward understanding the interactions between innate immune cells, such as macrophages, and bacterial PAMPs, relatively little is known about the activity of PAMPs present on fungi. Identification of the molecular mechanisms and the components responsible for internalization of β -glucans and their impact on inflammatory signaling should lead to a better understanding of innate immune responses to fungi. Accordingly, the present investigations were performed to define molecular mechanisms and components mediating β -glucan internalization and signaling by macrophages. We also investigated the

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role of dectin-1 and Toll-like receptor 2 (TLR2) receptors in internalization and signaling responses to purified fungal β -glucans.

MATERIALS AND METHODS

General reagents. Endotoxin-free buffers and reagents were scrupulously used in all experiments. Fungal β -glucan particles (derived from *S. cerevisiae*) were purchased from Sigma Chemical Co. (St. Louis, MO). The sizes of these particles predominantly ranged from 2 to 8 μ m. LPS from *Escherichia coli* 026:B6, as well as a 5-[[4,6-dichlorotriazin-2-yl] amino]-fluorescein hydrochloride-Celite complex (DTAF), nystatin, cytochalasin D, and other general reagents were also obtained from Sigma, unless indicated otherwise. Murine RAW 264.7 macrophages were purchased from ATCC and routinely cultured in Dulbecco modified Eagle medium containing 10% fetal bovine serum, 2 mM L-glutamine, penicillin (10,000 U/liter), and streptomycin (1 mg/liter). Cells were routinely passaged for no more than 6 weeks, discarded, and replaced with frozen stocks. Monoclonal antibody m2A11, which recognizes the dectin-1 receptor, was generously provided by Gordon Brown, University of Cape Town, South Africa (4). Also, a V5 epitope-tagged wild-type dectin-1 vector was provided by David Underhill, Institute for Systems Biology, Seattle, WA (6). Soluble glucan phosphate, which antagonizes binding of glucan particles to dectin-1 receptors, was a gift from David L. Williams, East Tennessee State University, Johnson City (16). TLR2^{-/-} mice were donated by Shizuo Achira, Research Institute for Microbial Disease, Osaka University, Osaka, Japan (21).

Generation of fluorescent β -glucan particles. To visualize the internalization of β -glucan by macrophages, *S. cerevisiae* β -glucan particles were coupled to the fluorophore DTAF. This was accomplished by adding 10 mg of DTAF dissolved in 0.1 M borate buffer (pH 7.0) to 25 mg of β -glucan particles suspended in 0.1 M borate buffer. The mixture was allowed to react overnight at room temperature with gentle stirring. Uncoupled DTAF was removed by extensive washing with phosphate-buffered saline (PBS). Labeled *S. cerevisiae* β -glucan particles were collected by centrifugation, dried, and weighed. After treatment with polymyxin, the final preparation was assayed for endotoxin. Labeled particles yielded fluorescence in the green range.

We scrupulously excluded endotoxin as the source of cellular responses to our β -glucan preparations. To do this, the β -glucan preparations were tested after each of the final washes for soluble endotoxin using a standard amoebocyte lysate assay with a low level of sensitivity, 0.125 IU (international unit)/ml (quantitative chromogenic *Limulus* amoebocyte lysate; BioWhittaker, Walkersville, MD). In addition, since β -glucans can trigger the *Limulus* amoebocyte lysate reaction, the preparations were also assayed with the Pyrostat assay (Associates of Cape Cod Incorporated, East Falmouth, MA), which is specific for bacterial endotoxin and which has a lower limit of sensitivity, 0.25 EU/ml. Only preparations with endotoxin levels below these thresholds were employed. Furthermore, we previously determined that endotoxin-resistant lung cells, which lack TLR4 signaling, can still respond to our particulate β -glucan preparations, indicating that β -glucan responses are independent of LPS (14). Thus, we are very confident that the responses observed reflected responses to β -glucan rather than responses to any contaminating endotoxin.

Phagocytosis assays and inhibitor treatments. To evaluate the mechanisms of macrophage internalization of β -glucan particles, we performed phagocytosis assays in the absence and presence of inhibitors of various uptake pathways. Our previous studies revealed parallel responses of fungal β -glucans interacting with native alveolar macrophages and with the RAW murine macrophage cell line (14). RAW 264.7 macrophage cells, thus, provide an efficient model system to study interactions of fungal cell wall components with macrophages. In addition, in our experience RAW cells exhibit somewhat less autofluorescence than native macrophages, making them useful for fluorescence microscopy studies. Furthermore, the RAW cell line exhibits substantially better transfection efficiency than native alveolar macrophages. For these reasons, we used these cells for most experiments in this study. RAW macrophages were pretreated with cytochalasin D (2 μ M) or nystatin (5 μ g/ml) for 30 min and throughout subsequent incubation with DTAF-labeled β -glucan. After various times, particle uptake was terminated by washing cells with ice-cold PBS, and unbound particles were removed by acid stripping (Hanks balanced saline solution buffered to pH 3.5), followed by fixation with 2% paraformaldehyde. Macrophage uptake of DTAF-glucan was visualized by fluorescence microscopy. XTT {2,3-bis(2-methoxy-5-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide} assays revealed no adverse effect of these uptake inhibitors on RAW cell viability at the concentrations employed (7).

To evaluate the potential role of the dectin-1 receptor in mediating internal-

ization and signaling from purified β -glucan particles, we treated cells with either monoclonal antibody m2A11 or glucan phosphate (3). RAW macrophages were placed on ice and incubated with either 100 μ g/ml of m2A11 or glucan phosphate for 45 min. After incubation, cells were allowed to bind DTAF-labeled β -glucan particles for 10 min on ice and then incubated at 37°C for 30 min. The RAW macrophages were gently washed and incubated at 37°C for the times indicated below. Particle uptake was determined as described above. To determine the role of TLR2 in mediating uptake of DTAF-labeled β -glucan, alveolar macrophages were collected from TLR2^{-/-} mice (21) and strain-matched wild-type controls by whole lung bronchoalveolar lavage (C57BL/6), and phagocytosis experiments were performed as previously described (25). We obtained ~200,000 alveolar macrophages per mouse. In preliminary studies, mouse native alveolar macrophages exhibited responses parallel to those of rat macrophages in our previous studies (24, 25).

Immunofluorescence studies for markers of endocytosis and NF- κ B activation. RAW macrophages were cultured on glass coverslips and stimulated with β -glucan particles. After stimulation and fixation, the macrophages were permeabilized with blocking buffer containing 0.05% saponin and 2 mg/ml bovine serum albumin for 30 min. Primary antibodies recognizing either the transferrin receptor (1 μ g/ml; eBioscience, Inc., San Diego, CA), an early endocytic marker, or lysosomal membrane protein 1 (Lamp-1; 1 μ g/ml; Research Diagnostics, Inc., Flanders, NJ) were diluted in blocking solution containing 0.05% saponin for 30 min, rinsed three times with the same solution, and incubated with a 1:100 dilution of Texas Red secondary conjugated antibodies for 30 min at room temperature. For actin filament visualization, cells were fixed as described above, permeabilized in 0.1% Triton X-100 in PBS, and stained with Texas Red-phalloidin (1:100; Molecular Probes, Eugene, OR) for 30 min. In additional experiments, the cells were subsequently stained for nuclear translocation of the p65 NF- κ B component as previously described (14). Nuclear counterstaining was performed with 4',6-diamidino-2-phenylindole (DAPI) (0.1 mg/ml; Sigma). Following extensive washing, the coverslips were mounted on glass slides and analyzed by fluorescence microscopy.

Conventional fluorescence microscopy was performed using an IX70 Olympus microscope equipped with filter packs. Fluorescein isothiocyanate and Texas Red labeling was observed under the fluorescence microscope using optics appropriate for these fluorophores (excitation at 470/40 nm and emission at 540/40 nm for fluorescein isothiocyanate and excitation at 540/25 nm and emission at 620/60 nm for Texas Red). Nuclear counterstaining with DAPI was observed at an excitation wavelength of 360/40 nm and an emission wavelength of 460/50 nm. In any given experiment, all photomicrographs were exposed and printed in the same way. Quantitative image analysis was performed using the "Metamorph" image-processing software (Universal Imaging Corp.) as previously described (17, 18).

Macrophage release of TNF- α following stimulation with fungal β -glucan particles. To evaluate subsequent macrophage inflammatory activation by β -glucans, we measured TNF- α release in the absence or presence of the various uptake inhibitors. RAW macrophages were grown in 96-well plates overnight at a concentration of 2×10^5 cells/well. Cells were pretreated for 30 min with uptake inhibitors before stimulation with β -glucan particles (100 μ g/ml). After a subsequent 8 h of incubation, the medium supernatants were collected and clarified by centrifugation. Samples were analyzed using a mouse TNF- α enzyme-linked immunosorbent assay (eBioscience, San Diego, CA) according to the manufacturer's instructions.

Dectin-1 phosphorylation following β -glucan challenge. RAW macrophages (5×10^6 cells) were transfected with 3 μ g of a V5 epitope-tagged wild-type dectin-1 plasmid using Nucleofector cell line solution V (Amaya Biosystems, Gaithersburg, MD) according to the manufacturer's instructions. All experiments were performed 24 h after transfection. RAW cells expressing V5 epitope-tagged wild-type dectin-1 were treated for 30 min with 50 μ M MG132 to prevent proteasomal degradation of receptors prior to the addition of β -glucan particles (100 μ g/ml) suspended in a solution containing 1 mM sodium *ortho*-vanadate for 15 min. Cells were then disrupted with lysis buffer (1% Triton X-100, 10 mM Tris, 50 mM NaCl, 0.2 mM sodium *ortho*-vanadate, pH 7.4, and protease inhibitors) for 30 min on ice. Samples were then centrifuged at 14,000 rpm at 4°C, and the supernatants were collected. Protein concentrations in the lysates were determined by a Coomassie blue protein assay (Pierce Chemical Co., Rockford, IL) using bovine serum albumin standards as references. Lysates were separated by nonreducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and blotted with antibody to the polyclonal anti-V5 antibody (1 μ g/ml; Invitrogen, Carlsbad, CA). To determine receptor tyrosine phosphorylation, the lysates were immunoprecipitated with antibody to phosphotyrosine residues. Following preclearing, the lysates were incubated with phosphotyrosine antibody (4 μ g/ml; Upstate Biotechnology, Lake Placid, NY) on

ice for 15 min. Complexes were precipitated with protein G Sepharose beads overnight at 4°C. The beads were then washed twice with lysis buffer and boiled for 3 min in nonreducing Laemmli buffer. Tyrosine-phosphorylated proteins were separated on 10% polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were blocked with 5% milk in Tris-buffered saline and incubated with phosphotyrosine antibody (1 µg/ml; Santa Cruz Biotechnology, Santa Cruz, CA). Subsequently, the membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibody and detected by the ECL chemiluminescence detection system (Amersham Biosciences).

Statistical analysis. All data were expressed as means $\pm$ standard errors of the means. Differences for multigroup comparisons were initially assessed by analysis of variance. Differences between specific groups were determined using a two-tailed Student *t* test. Statistical testing was performed using an SPSS/JMP software program, and statistical differences were considered significant if the *P* value was <0.05 .

RESULTS

Fungal β -glucan particles bind RAW 264.7 macrophages and are internalized. Macrophages are phagocytic cells that possess various receptors and internalization mechanisms uniquely adapted for the uptake of specific types of particles. The response to any given particle depends on the specific combination of receptors, internalization mechanisms, and subsequent signaling cascades employed. We, therefore, investigated the kinetics and mechanisms of *S. cerevisiae* β -glucan particle internalization by macrophages. RAW 264.7 macrophages were incubated with DTAF-labeled β -glucan particles for increasing times (Fig. 1). Following incubation, the cells were acid stripped to remove loosely bound and noninternalized particles. The particles, which appeared to be in the 2- to 8-µm size range as determined by light microscopy, were considered internalized if there was clearly a complete rim of surrounding cytoplasm. Fluorescence microscopy demonstrated that there was association of particles with the outer surfaces of cells within minutes. However, the majority of particles were not completely internalized until after 1 to 2 h of incubation. To determine whether similar kinetics occurred in native macrophages and the process was not just a function of the cell line studied, we performed an additional experiment utilizing native rat alveolar macrophages that were obtained by bronchoalveolar lavage as previously described (24, 25). The kinetics of particle internalization did not vary significantly between RAW cells and native alveolar macrophages (data not shown).

Internalized β -glucan particles are associated with markers of phagosome formation. We next used antibodies specifically recognizing transferrin receptor and Lamp-1 as membrane markers to determine interactions of internalized β -glucan particles with compartments of the endocytic pathway. Transferrin receptor, a marker for early endocytic pathways, demonstrated staining around internalized β -glucan particles (Fig. 2A). Staining for Lamp-1, a marker of the late endocytic pathway, also revealed a time-dependent increase in the appearance of a fluorescent halo surrounding β -glucan particles. All ingested particles were found to have both transferrin receptor and Lamp-1 surrounding them, which was still visible even after 4 h of incubation (Fig. 2B).

Internalization of β -glucan particles is dependent on actin cytoskeleton polymerization but not on caveolar mechanisms. Phagocytosis of particles frequently requires actin polymerization (1, 7, 8). However, additional studies have indicated that

caveolar uptake mechanisms can function in the uptake of certain infectious agents, including specific viruses and certain bacteria (10, 11, 15). We therefore utilized inhibitors to target these specific pathways and evaluate their relative contributions to macrophage uptake of fungal β -glucan particles. RAW macrophages were incubated with DTAF-labeled β -glucan particles for 4 h in the presence of these inhibitors, and the numbers of internalized particles were determined (Fig. 3A). Nystatin, a drug that binds to cholesterol and causes caveolae to flatten, thereby inhibiting uptake through this mechanism, did not alter the number of internalized particles from the number observed for the control group. In contrast, cytochalasin D, an antagonist of actin polymerization, significantly decreased particle internalization by 90% ($P < 0.05$ compared to particle uptake in the absence of inhibitor). These findings were determined in two independent experiments, and in each experiment at least 250 contiguous cells were visualized. In addition, neither nystatin nor cytochalasin D caused any alteration in RAW macrophage viability. Taken together, these findings indicate that internalization of β -glucan particles is dependent on actin cytoskeleton polymerization but does not involve interactions with components of caveolar uptake pathways.

Macrophage inflammatory activation is not dependent on β -glucan particle internalization. To determine whether inhibition of β -glucan internalization also alters macrophage inflammatory signaling, we evaluated the effect of pharmacologically inhibiting β -glucan internalization and subsequent nuclear translocation of p65 NF- κ B and production of TNF- α . We previously reported that cell wall β -glucans derived from *Pneumocystis* induce p65 NF- κ B activation by 2 h and that this effect is sustained for up to 6 h after the onset of β -glucan challenge (7). We therefore evaluated p65 NF- κ B nuclear translocation in RAW macrophages challenged with β -glucan in the presence of nystatin and cytochalasin D. Cells were pretreated with these inhibitors and incubated with β -glucan particles for 2 h, and p65 NF- κ B was localized by fluorescence microscopy. NF- κ B activation was plotted as mean nuclear intensities for the various treatment groups (Fig. 4A). Treatment with cytochalasin D, a potent antagonist of particle uptake through actin-dependent mechanisms, did not significantly alter fungal β -glucan-induced p65 NF- κ B nuclear translocation. Although treatment with nystatin resulted in a slight reduction in p65 NF- κ B nuclear translocation, this translocation was not significantly reduced compared to that observed for untreated RAW cells challenged with β -glucan particles in the absence of inhibitor. These data strongly indicate that β -glucan particle internalization through actin-mediated mechanisms is not necessary for the initiation of inflammatory signaling pathways in macrophages.

We further evaluated the ability of β -glucan particles to stimulate macrophage release of TNF- α , a prototypic inflammatory cytokine, under conditions under which internalization was impaired. Consistent with our previous observations, RAW macrophages were stimulated to release TNF- α following challenge with β -glucans (7). We also observed that TNF- α production was not altered by inhibition of actin internalization pathways (Fig. 4B). Furthermore, TNF- α release was not altered in the presence of nystatin. To further exclude a defect in cytokine secretion, RAW cells were also stimulated with LPS

FIG. 1. Internalization of fungal β -glucan particles. RAW macrophages were treated with DTAF-labeled β -glucan particles (10 particles/cell) for the times indicated. Cells were washed, fixed, and viewed by fluorescence microscopy. Although β -glucans bind rapidly, complete uptake of the particles required 1 to 2 h of incubation.

FIG. 2. Internalized β -glucan particles associate with early and late phagosome markers. RAW macrophages were incubated with DTAF-labeled β -glucan particles (10 particles/cell). Panels A and D are phase-contrast micrographs, panels B and E show staining of β -glucan particles in green and the phagosome markers in red (panel B shows the transferrin receptor, and panel E shows Lamp-1), and panels C and F show the areas indicated by the boxes in panels B and E magnified. In panels A, B, and C the macrophages were stained with antibody to the transferrin receptor, an early phagosome marker, following 60 min of incubation with the particles. The β -glucan particles (green) were internalized and became surrounded by the transferrin receptor, an early phagosome marker (red halo) (arrows). In panels D, E, and F the macrophages were further stained with anti-Lamp-1 antibody, a late phagosome marker, after 4 h of incubation with the particles. Again, the β -glucan particles (green) were internalized and became surrounded by Lamp-1 associated with internalized β -glucan particles (red halo) (arrows).

(0.1 μ g/ml) in the presence of these agents. Neither cytochalasin D nor nystatin altered the ability of RAW macrophages to signal, as shown by LPS-induced TNF- α release in the presence of these inhibitors (data not shown).

Dectin-1 but not TLR2 mediates binding and internalization of purified β -glucan particles by macrophages. Various receptors have been implicated in mediating the β -glucan effects on macrophages. Two of these receptors, dectin-1 and TLR2, have been investigated recently, and some studies have suggested that there are collaborative interactions between these two receptors (6). To first evaluate the role of TLR2 in mediating β -glucan internalization, we harvested alveolar macrophages from TLR2^{-/-} mice and challenged these cells with fungal β -glucan particles. Phalloidin staining of F-actin was used to identify the accumulation of actin filaments consistent with phagocytic cups surrounding the β -glucan particles (Fig. 5). The binding of fungal β -glucan particles and the formation of phagocytic cups were not significantly different for TLR2^{-/-} and wild-type control macrophages during 60 min of incubation. Furthermore, alveolar macrophages from TLR2^{-/-} mice produced just as much TNF- α as wild-type control macrophages produced following β -glucan stimulation over 8 h. While wild-type C57BL/6 macrophages produced $32,045 \pm 11,097$ pg/ml of TNF- α following stimulation with β -glucan, alveolar macrophages from TLR2^{-/-} mice produced $30,800 \pm 11,693$ pg/ml of the cytokine (not significantly different).

Hence, these data do not support a dominant independent role for TLR2 in mediating uptake or macrophage activation in response to purified fungal β -glucans.

In contrast, dectin-1 has recently been shown to participate

FIG. 3. Actin depolymerization suppresses β -glucan internalization. RAW macrophages were treated with nystatin (5 μ g/ml) or cytochalasin D (CyD) (2 μ M) for 30 min prior to challenge with DTAF-labeled β -glucan particles (10 particles/cell) and during a subsequent 4-h incubation. Cells were washed, acid stripped, mounted on slides, and visualized by fluorescence microscopy. The number of particles per cell was determined by using at least 250 cells in each of two independent experimental runs. The data are means and standard errors. An asterisk indicates that the P value is <0.05 for a comparison with control uptake with no inhibitor.

in phagocytosis and proinflammatory signaling elicited by macrophages challenged with intact yeast and zymosan, a yeast cell wall fraction composed of both mannoproteins and β -glucans (2). Therefore, we were interested in evaluating the location of dectin-1 during interactions of purified fungal β -glucan cell wall components with macrophages (Fig. 6). Dectin-1 receptors were recruited to areas of β -glucan particle binding and uptake within minutes of challenge, and they were consistently localized to areas of phagocytic cups on the macrophage (Fig. 6). Furthermore, actin cytoskeleton disruption by cytochalasin D did not affect dectin-1 recruitment to regions of the macrophage surface membrane interacting with the β -glucan particles. Thus, we concluded that dectin-1 recruitment to β -glucan particles does not depend on the actin cytoskeleton.

Dectin-1 is phosphorylated and participates in uptake and macrophage activation in response to purified fungal β -glucans. Dectin-1 receptors contain a cytoplasmic immunoreceptor tyrosine-based activation-like motif. Such motifs have been shown to be involved in recruitment and binding of signaling molecules. To address whether dectin-1 interactions with β -glucans lead to receptor activation, we assessed the tyrosine phosphorylation state of dectin-1 receptors in RAW macrophages before and after stimulation with β -glucan particles (Fig. 7). The total amount of the recoverable dectin-1 receptor was slightly reduced following glucan treatment, likely due to interactions with the particles. However, tyrosine phosphorylation of dectin-1 was evident as early as 15 min following fungal β -glucan stimulation, indicating that there was receptor activation following interaction with the purified fungal cell wall component.

Finally, studies were undertaken to antagonize binding of β -glucan with dectin-1 receptors and to assess the impact on both particle uptake and associated cytokine release. To accomplish this, dectin-1 was alternately inhibited with either a monoclonal antibody recognizing the receptor (m2A11) or with glucan phosphate, a soluble β -glucan competitive carbohydrate antagonist (Fig. 8A). Both agents significantly decreased the uptake of fungal β -glucan particles. In addition, TNF- α secretion was also substantially antagonized by the inhibition of dectin-1 binding to the β -glucan particles, either with m2A11 or with soluble glucan phosphate (Fig. 8B). To further exclude any nonspecific immunoglobulin effect, we also performed control experiments with nonimmune immunoglobulin G (IgG). Nonimmune IgG did not have any significant activity in altering β -glucan-stimulated TNF- α release. Specifically, the relative TNF- α release from β -glucan-stimulated RAW macrophages yielded a $100.0\% \pm 6.4\%$ maximal response in the absence of antibody, compared to the $120.7\% \pm 7.0\%$ maximal response in the presence of nonimmune IgG (100 μ g/ml) (not statistically different). Thus, the effects observed with antibody m2A11 appeared to be specifically related to the effect of m2A11 on dectin-1 rather than to any nonspecific immunoglobulin activity. The greater decrease in particle internalization observed with glucan phosphate could have been due to binding of receptors other than dectin-1. Together, these data indicate that dectin-1 appears to be required for efficient β -glucan particle internalization and also in the signaling of cytokine release.

FIG. 4. Actin-dependent internalization of fungal β -glucan particles is not necessary for inflammatory signaling. RAW cells were treated with nystatin (5 μ g/ml) or cytochalasin D (CyD) (2 μ M) prior to stimulation with β -glucan particles (100 μ g/ml). (A) Nuclear translocation of p65 NF- κ B was visualized by immunostaining with a specific antibody recognizing p65 and a Texas Red-conjugated secondary antibody after 2 h. Quantitative differences in fluorescence intensities between nuclei and cytoplasm were calculated and plotted. Inhibition of actin-dependent internalization with cytochalasin D did not alter NF- κ B activation. (B) Release of TNF- α into the medium was determined by an enzyme-linked immunosorbent assay after 8 h of incubation with fungal β -glucan in the presence or absence of the same inhibitors. Again, cytochalasin D did not alter TNF- α release. An asterisk indicates that there was no significant difference for a comparison of macrophages stimulated with β -glucan in the absence of any inhibitor (i.e., the second bar in each graph) and macrophages stimulated with β -glucan in the presence of inhibitors. The data are the means and standard errors of the means obtained from three experiments.

DISCUSSION

Macrophages are the principal phagocytic component of innate immunity in the lung. Upon contact with fungal pathogens, β -glucans on the surface of the fungal cell wall activate macrophages to release inflammatory mediators. We recently established that the opportunistic fungal pathogen *Pneumocystis* induces macrophage inflammatory activation through

FIG. 5. TLR2^{-/-} alveolar macrophages are capable of binding and internalizing fungal β -glucans. Alveolar macrophages were harvested from TLR2^{-/-} and wild-type C57Bl/6 control mice. Macrophages were cultured on coverslips and incubated with unlabeled β -glucan particles for 1 h. The preparations were then fixed, permeabilized, and stained for F-actin filaments using Texas Red-phalloidin. The arrows indicate accumulation of actin filaments consistent with phagocytic cups surrounding β -glucan particles. The binding of fungal β -glucan particles and the formation of phagocytic cups were not significantly different for TLR2^{-/-} and wild-type control macrophages.

NF- κ B signaling by utilizing mechanisms distinct from those utilized during LPS stimulation. The kinetics of the macrophage NF- κ B response to β -glucans showed both delayed and sustained activation compared to LPS (7). Therefore, we sought to obtain additional insights into the mechanisms of internalization of β -glucan particles and its potential effect on signaling. The current investigation revealed that internalization of fungal β -glucan particles is actin dependent, but we also demonstrated that internalization is not required for particle-induced inflammatory signaling. We also observed that decin-1 interactions, but not TLR2, are necessary both for efficient internalization and for macrophage cytokine release following β -glucan stimulation.

The nature of the pathogen interacting with macrophages determines the distinct phagocytic mechanisms employed, as well as the related activation of inflammatory signaling cascades. Actin reorganization, one of the first molecular events involved in phagocytosis, is required for membrane ruffling,

phagosome formation, and phagolysosome maturation (1, 7, 8). The current study demonstrated that actin polymerization at the site of contact between β -glucan particles and macrophages is necessary for phagocytosis. This was confirmed by the marked inhibition of fungal β -glucan particle uptake in the presence of cytochalasin D, a drug that induces depolymerization of actin microfilaments. These findings are in complete agreement with previous studies that showed that actin polymerization is required for internalization of intact yeast and zymosan, a cell wall fraction composed of β -glucans and mannans (12, 26). In contrast, inhibition of caveolar uptake pathways by nystatin did not have any effect on β -glucan particle uptake or macrophage inflammatory signaling.

β -Glucan particles are purified fractions of fungal cell walls. Although these preparations generally range in size from 2 to 8 μ m, they contain variable amounts of smaller particles visible by light microscopy. These preparations also exhibit differences in particle size and relative biological activity depending

FIG. 6. Dectin-1 recruitment to macrophage membrane regions interacting with fungal β -glucan particles. RAW macrophages were transfected with V5 epitope-tagged dectin-1 receptor, stimulated with fungal β -glucan particles for 15 min, and stained with fluorescein isothiocyanate-conjugated anti-V5 antibody. Panels A and C are phase-contrast images, while panels B and D show the fluorescence of the dectin-1 receptor. Dectin-1 recruitment occurred within minutes of contact between β -glucan particles and cells (A and B). Inhibition of actin polymerization (C and D) did not affect dectin-1 recruitment to the β -glucan particles. The arrows indicate well-formed surface-localized phagocytic cups containing the dectin-1 receptors.

on the fungal species from which they are derived. Considerable evidence indicates that β -glucans are potent immune stimulatory agents (9, 14, 25). Our preliminary studies of macrophage NF- κ B activation indicated that on average, 4.85 ± 3.22 particles are associated with each activated cell over the first hour. However, we have also clearly observed NF- κ B activation in macrophages that do not contain visibly internalized β -glucan particles following acid stripping, lending further support to the hypothesis that particle interactions with the cell membrane, rather than particle uptake, are necessary for cell activation. Depending on the host cell and conditions, β -glucans have been shown to interact with a variety of cell surface receptors, including CD11b/CD18, lactosylceramide, Toll receptors, and dectin-1 (1, 2, 9, 14, 25). The overall biological effects of any given β -glucan preparation on a particular host cell likely reflect the size and origin of the glucan, which receptor or receptors are available on the cell for interactions,

FIG. 7. Dectin-1 interactions with β -glucan particles results in tyrosine phosphorylation of the receptor. RAW macrophages were transfected with V5 epitope-tagged dectin-1 and stimulated with fungal β -glucan particles for 15 min, and lysates were prepared. (Top panel) Total lysates were immunoblotted with an antibody recognizing the V5 epitope tag. Incubation of the macrophages with β -glucan particles even in the presence of proteasomal inhibitors resulted in a slight reduction in recoverable dectin-1 receptor. (Bottom panel) Sequential immunoprecipitation and immunoblotting with an antiphosphotyrosine antibody demonstrated that dectin-1 receptors are phosphorylated following interaction with fungal β -glucan particles.

FIG. 8. Dectin-1 is necessary for efficient internalization of β -glucan particles and subsequent inflammatory signaling. RAW macrophages were treated with either m2A11 (100 μ g/ml) or glucan phosphate (GluP) (100 μ g/ml) and stimulated with DTAF-labeled β -glucan particles. (A) Internalization of particles was measured after 4 h by fluorescence microscopy and counting. (B) Secretion of TNF- α into the medium was measured after 8 h by an enzyme-linked immunosorbent assay. Both the anti-dectin-1 antibody m2A11 and the soluble glucan phosphate carbohydrate antagonist significantly reduced the internalization of β -glucan particles and the subsequent release of TNF- α . An asterisk indicates that the P value is <0.05 for a comparison with control uptake with no inhibitor.

and the availability of additional host proteins that may bind and modulate β -glucan activity (23).

Accumulating data indicate that Toll receptors participate in inflammatory signaling in macrophages in response to both intact yeast and zymosan. We previously demonstrated that β -glucan-induced TNF- α secretion from macrophages was partially mediated by MyD88, a Toll receptor adaptor protein (7). Additional studies indicated that TLR2 receptors are recruited to phagolysosomes containing zymosan but that their basal surface expression is quite low (22). Our data demonstrate that TLR2 is not a necessary component for purified β -glucan internalization or inflammatory signaling in RAW macrophages. Alveolar macrophages from TLR2 $^{-/-}$ mice exhibited normal phagocytosis of β -glucan particles and potent β -glucan-induced TNF- α secretion equivalent to that of wild-type macrophages. Taken together, these data indicate that while TLR receptors and signaling components may be involved in macrophage responses, other receptors and pathways can compensate and transmit β -glucan-induced responses in the absence of TLR2.

Dectin-1 has also been shown to mediate biological effects from intact yeast and zymosan interacting with macrophages (3). In the current investigation we evaluated the interaction of

dectin-1 with purified β -glucan particles, which have been shown to promote inflammatory responses in macrophages. Interactions of dectin-1 were necessary for both efficient macrophage internalization and inflammatory activation in response to fungal β -glucans. Interestingly, glucan phosphate suppressed internalization more efficiently than the m2A11 monoclonal antibody against dectin-1. It is still possible that glucan phosphate, as a soluble β -glucan carbohydrate, can interact with multiple receptors involved in the recognition and uptake of β -glucans, including possibly CD11bCD18 (Mac-1), as well as the dectin-1 receptor (6).

In summary, this study demonstrated that internalization of fungal β -glucan particles derived from *S. cerevisiae* is a complex process requiring actin polymerization but not participation of components of the caveolar uptake pathways. Internalization of particles through actin-dependent mechanisms is not necessary for the induction of macrophage inflammatory responses to β -glucan particles. Furthermore, dectin-1 rather than TLR2 is an integral part of macrophage recognition of β -glucan particles, mediating both internalization and subsequent proinflammatory signaling in response β -glucan cell wall components.

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