Pneumocystis carinii Cell Wall Beta-Glucans Initiate Macrophage Inflammatory Responses through NF-κB Activation

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RUNNING TITLE: Pneumocystis carinii glucan activates NF-kB.
ABSTRACT

Beta-glucans (β-glucans) are major structural components of fungi. We have recently reported that the pathogenic fungus Pneumocystis carinii (P. carinii) assembles a β-glucan-rich cell wall that potently activates alveolar macrophages to release pro-inflammatory cytokines and chemokines. Purified P. carinii β-glucans predictably induce both cytokine generation and associated neutrophilic lung inflammation. Herein, we demonstrate that P. carinii β-glucan-induced macrophage stimulation results from activation of NF-κB. Although analogous to macrophage activation induced by bacterial lipopolysaccharide (LPS), P. carinii β-glucan-induced macrophage NF-κB activation exhibits distinctly different kinetics, with slower induction and longer duration compared to LPS stimulation. Macrophage activation in response to P. carinii β-glucan was also substantially inhibited with the NF-κB antagonist pyrrolidine dithiocarbamate. In addition to different kinetics of NF-κB activation, P. carinii β-glucan and LPS also utilize different receptor systems to induce macrophage activation. Macrophages from TLR-4 deficient and wild type mice produced equivalent amounts of TNFα when stimulated with P. carinii β-glucan. However, TLR-4 deficient macrophages were refractory to stimulation with LPS. In contrast, MyD88 deficient macrophages exhibited a significant (though partial) blunted response to P. carinii β-glucan. These data demonstrate that P. carinii β-glucan acts as potent inducer of macrophage activation through NF-κB utilizing cellular receptors and signaling pathways distinct from LPS.
INTRODUCTION

*Pneumocystis carinii* (*P. carinii*) remains a common cause of pneumonia among individuals with compromised immune function, resulting in substantial morbidity and mortality in patients with AIDS, malignancies, and following organ transplantation (1,2). Similar to other related fungi, *P. carinii* assembles a β-glucan rich cell wall comprised of glucose residues arranged in β-(1,3)-D-glucopyranosyl polymers with β-(1,6)-D-glucopyranosyl side chains of varying length and frequency distributions (3-5). We recently reported the isolation and characterization of a particulate carbohydrate cell wall fraction derived from *P. carinii*, which is composed largely of β-glucans (6,7). In addition to providing structural support to the organism, *P. carinii* cell wall β-glucans potently induce inflammatory activation of lung cells, with release of pro-inflammatory cytokines and chemokines during this infection (6,7).

Although essential for host responses and clearance of *P. carinii*, alveolar macrophage induced lung inflammation contributes substantially to respiratory failure and death during *P. carinii* pneumonia (8). Indeed, exuberant host inflammatory responses to this organism stimulate alveolar edema and neutrophilic infiltration, resulting in acute lung injury and respiratory failure. Pulmonary inflammation exerts a greater effect on clinical outcome than direct actions of the organism (9). This concept is supported by the clinical observation, that severe *P. carinii* pneumonia remains the only form of acute lung injury that has clearly been demonstrated to improve with agents which non-specifically down-regulate host inflammatory responses, namely corticosteroid therapy (10). Thus, understanding the mechanisms of host inflammatory
cell activation by *P. carinii* is of critical importance and may provide essential insights into means by which the inflammatory response may be selectively modulated to advantage the host.

Particulate β-glucans from fungal organisms interact with cognate receptors on macrophages stimulating the release of reactive oxidants, eicosanoid metabolites, cytokines and chemokines (11). In an analogous fashion, whole *P. carinii* organisms, as well as β-glucan isolates purified from *P. carinii*, both strongly promote inflammatory activation of alveolar macrophages with subsequent release of tumor necrosis factor-alpha (TNFα) and macrophage inflammatory protein-2 (MIP-2) (6,12). *P. carinii* initiated macrophage stimulation is mediated through ligation of glucan receptors and is abolished by digestion with β-glucanase, indicating that glucan components of the organism are chiefly responsible for macrophage inflammatory responses during this infection (6,12). Although these investigations provide important insight into initiation of *P. carinii* induced lung inflammation, signal transduction mechanisms mediating macrophage activation during *P. carinii* challenge have not yet been defined.

Nuclear factor (NF)-κB is a ubiquitous transcription factor, which regulates inflammatory gene expression in many immune effector cells. NF-κB activation is induced by a variety of extracellular signals such as proinflammatory cytokines, bacterial lipopolysaccharide and viral products (13-16). In quiescent unstimulated cells, NF-κB is composed of homodimers or heterodimers containing various Rel proteins, including NF-κB1 (p50), NF-κB2 (p52), Rel A (p65), Rel B and c-Rel, complexed to an inhibitory anchor protein termed I-κBα (17).
Following stimulation with an appropriate agent, I-κBα is phosphorylated and rapidly degraded through proteosomal mechanisms (17). Removal of I-κBα permits translocation of active NF-κB components to the nucleus where they initiate cytokine gene transcription (17,18).

In addition, receptor mechanisms participating in innate macrophage immune responses to β-glucans are not yet characterized. The Toll family of immune receptors represents a conserved family of pattern recognition receptors, which respond to microbial products and other agonists to trigger host inflammatory responses (19). For instance, Toll-like receptor 4 (TLR-4) is a well-defined mechanism conferring host cell response to bacterial LPS (20). Furthermore, many, though not all, Toll family responses also require activity of the MyD88 adaptor protein to initiate cell signaling (21,22). Cellular activation through such innate immune receptors has not been extensively studied during challenge of cells with fungal cell wall β-glucans.

The branching structures and biological activities of fungal β-glucans vary considerably with the sources from which they are derived. Zymosan, a commonly studied β-glucan source prepared from the non-pathogenic fungus *Saccharomyces cerevisiae* (*S. cerevisiae*) and other soluble non-pathogenic glucans activate nuclear factor NF-κB in cell lines (23-26). Whether NF-κB participates in signaling macrophage inflammatory activation to release TNFα following stimulation with cell wall β-glucans derived from *P. carinii*, and whether such signaling can be modified pharmacologically is currently unknown. The present investigation was, therefore, performed to determine the role of NF-κB in inflammatory activation of macrophages challenged *P. carinii* β-glucans. We further tested the roles of TLR-4 and MyD88 in signaling macrophage responses to *P. carinii* β-glucans, and whether macrophage activation could be
modified with pyrrolidine dithiocarbamate (PDTC), a potent inhibitor of NF-κB.
MATERIALS AND METHODS

Reagents. Endotoxin-free buffers and reagents were scrupulously used in all experiments. LPS from Escherichia coli 026:B6, pyrrolidine dithiocarbamate (PDTC) and other general reagents were obtained from Sigma Chemical Co. (St. Louis, MO), unless otherwise specified. *P. carinii* was derived originally from the American Type Culture Collection stock (ATCC, Manassas, VA) and has been passaged in our immunosuppressed rat colony (8). Murine RAW 264.7 macrophages were also purchased from ATCC and routinely cultured in DMEM containing 10% fetal bovine serum and 2 mM L-glutamine, penicillin 10,000U /L, and streptomycin 1mg /L.

**Generation of a Pneumocystis carinii β-Glucan-Rich Cell Wall Isolate.** All animal experimentation was reviewed and approved by the Mayo Institutional Animal Care and Usage Committee. A glucan rich cell wall fraction from *P. carinii* was prepared as we recently described (6,7). *P. carinii* pneumonia was induced in dexamethasone treated immunosuppressed Harlan Sprague Dawley rats (Harlan, Inc., Indianapolis, IN) (8). *P. carinii* organisms were isolated from lungs of heavily infected animals by homogenization and filtration through 10-µm filters. The organisms were autoclaved (120°C, 20 minutes), disrupted by ultrasonication (200W for 3 minutes, six times), and glucans isolated by NaOH digestion and lipid extraction as we previously detailed (6,7). The final product contained predominantly carbohydrate (95.7%) and released 82% of its content as D-glucose following hydrolysis (6). Extensive measures were employed to ensure that the fraction was free of endotoxin. Prior to use in culture, the *P. carinii*
cell wall fractions were washed with 0.1% SDS, and then vigorously washed with distilled physiological saline to remove the detergent. The final preparation was assayed for endotoxin with the Limulus amebocyte lysate assay method and found to consistently contain <0.125 units of endotoxin (6).

**Preparation of nuclear and cytosolic lysate fractions.** To evaluate I-κBα degradation and NF-κB activation, nuclear and cytosolic fractions were prepared from macrophages (RAW 264.7; 2x10⁶ cells/well) incubated in the presence or absence of particulate *P. carinii* β-glucans (2.5 x 10⁶ particles/ml) or LPS (0.1 μg/ml) in parallel cultures. At specified times, cells were scraped and suspended in 1 ml of buffer A (10 mM HEPES, 1.5 mM MgCl₂, 10 mM KCl, pH 7.9), freshly supplemented with 0.5 mM dithiothreitol, 10 μg/ml leupeptin, 2 μg/ml aprotinin, 2 μg/ml pepstatin and 1 μM phenylmethylsulfonyl fluoride. Cytosolic preparations were made by the addition of Lysis buffer (buffer A containing 0.1% NP-40) for 5 minutes, followed by centrifugation (6,500 rpm, 3 min, 4°C). The supernatants were collected as the cytosolic fractions. The remaining pellets were re-suspended in 15 μl of buffer B (20 mM HEPES pH 7.9, 25% v/v glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA), supplemented with protease inhibitors as above. Nuclear suspensions were incubated for 30 minutes at 4°C with intermittent agitation, then centrifuged (12,000 x g, 20 min, 4°C) to remove nuclear debris. Supernatants were collected and diluted with 30 μl of Buffer D (20 mM HEPES pH 7.9, 20% v/v glycerol, 0.05 M KCl and 0.2 mM EDTA), containing the inhibitors described above, and designated as the nuclear fractions. Cytosolic and nuclear fractions were stored at -70°C until assay. Protein
Concentrations in respective extracts were determined by coomassie protein assay (Pierce Chemical Company, Davenport, IA) referenced against a BSA standard.

**Determination of Cytosolic I-κBα.** To first determine the effect of *P. carinii* cell wall β-glucan stimulation on macrophage levels of IκB-α, immunoblot analysis was performed on cytosolic lysates prepared from RAW 264.7 macrophages stimulated with particulate *P. carinii* cell wall β-glucan. Cytosolic lysates from *P. carinii* β-glucan stimulated cells were compared to lysates prepared from LPS (0.1 µg/ml) treated macrophages. Lysates were separated on 10% polyacrylamide gels and transferred electrophoretically to nitrocellulose membranes. The membranes were blocked with 5% milk in Tris buffered saline and incubated with polyclonal antibody recognizing the carboxyterminus of IκB-α (200 µg/ml; Santa Cruz Biotechnology, Santa Cruz, CA) for 1-2 hours at room temperature. Subsequently, membranes were washed and incubated with HRP-conjugated secondary antibody and detected by a chemiluminescence detection system ECL (Amersham, Arlington Heights).

**Evaluation of NF-κB Activation.** To determine activation and nuclear translocation of NF-κB, electromobility shift assays (EMSA) were performed on nuclear lysates prepared from RAW 264.7 macrophages stimulated with either *P. carinii* β-glucan or LPS. A double-stranded DNA probe which binds NF-κB (5 AGT TGA GGG GAC TTT CCC AGC 3; Santa Cruz Biotechnology, Santa Cruz, CA) was radiolabeled with (γ^32P)-ATP and EMSA performed on 5 µg of nuclear extract as described previously (27). To confirm specificity of DNA binding, in some experiments a 50-fold excess of unlabelled NF-κB probe was added to the reaction for 15
minutes prior to addition of the radiolabeled NF-κB probe. In further studies, supershift analyses were performed using antibodies to each of the specified NF-κB subunits (p50 - 200 µg/0.1ml, p65 - 200 µg/ 0.1ml or c-Rel - 200 µg/ 0.1ml; Santa Cruz). These antibodies were incubated with nuclear extracts for 15 minutes prior to addition of radiolabeled DNA probe recognizing NF-κB. All EMSA reactions were subsequently incubated for 30 minutes at room temperature and DNA-protein complexes separated on 6% polyacrylamide gels and visualized by autoradiography.

**Fluorescence Visualization of NF-κB Nuclear Translocation.** To further define the time course of NF-κB activation following macrophage stimulation with *P. carinii* β-glucans, immunofluorescence was employed to detect translocation of the p65 component of NF-κB to the nucleus. RAW 264.7 cells were seeded onto sterile 22 x 22mm glass cover slips in 6-well tissue culture plates and cultured with *P. carinii* β-glucans or LPS for the indicated times prior to fixation with 2% paraformaldehyde for 10 minutes at 37°C. In parallel experiments, cells were treated for 30 min with the NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC; 10 µM) prior to addition of *P. carinii* β-glucan. After macrophage stimulation and fixation, the cells were subsequently permeabilized with methanol for 2 minutes at 22°C, and rinsed with PBS. Blocking of non-specific binding sites was performed with 5% goat serum in PBS for 30 minutes at room temperature. Goat anti-mouse p65 (1 µg/ml; Santa Cruz) was added and incubated for 60 minutes at room temperature. Cover slips were washed extensively and incubated for 60 minutes with a 1:50 dilution of Texas Red dye conjugated donkey anti-goat polyclonal antibody (Jackson ImmunoResearch Laboratories, New Grove, PA) or 1:1000 dilution of FITC conjugated rabbit anti-goat polyclonal antibody (ICN-Cappel, Costa Mesa,
CA). Nuclear staining was performed with 4,6-diamidino-2-phenylindole (DAPI; 0.1 µg/ml, Sigma). Following extensive washing, the cover slips were mounted on slides and analyzed by fluorescence microscopy.

Conventional fluorescence microscopy was performed using an IX70 Olympus microscope equipped with filter packs. FITC and TX Red labeling was observed under the fluorescence microscope using optics appropriate for these fluorophores (excitation = 470/40 nm; emission = 540/40 nm for FITC and excitation = 540/25 nm; emission = 620/60 nm for TX Red). Nuclear staining with DAPI was observed at excitation = 360/40 nm; emission = 460/50 nm. In any given experiment, all photomicrographs were exposed and printed identically. Quantitative image analysis was performed using the "Metamorph" image-processing program (Universal Imaging Corp.) as previously described (28,29).

**Role of TLR-4 and MyD88 in Macrophage TNFα Release Following *P. carinii* β-glucan Stimulation.** We have previously shown that isolated *P. carinii* cell wall β-glucans directly activate alveolar macrophages to release TNFα, a centrally important cytokine promoting lung inflammation during *P. carinii* pneumonia (6,12). To begin to evaluate the potential roles of Toll-like receptors and the MyD88 toll adaptor protein in mediating macrophage activation, alveolar macrophages were collected from TLR4 -/- mice (30), MyD88 -/- mice (31,32), and strain-matched wild type controls (C57Bl/6). Macrophages were recovered by BAL as previously described (6). Two x 10⁵ alveolar macrophages were plated per well in 96-well tissue culture plates, allowed to adhere for 60 minutes, and gently washed to remove any
unattached cells. Subsequently, varying *P. carinii* β-glucan concentrations (2.5 to 5 x 10^6 particles/ml) were incubated with the alveolar macrophages for 12 hours (37°C, 5% CO₂). Following incubation, the media was removed, centrifuged (10,000 x g for 5 minutes) to remove any particulate material, and assayed for the presence of TNFα by ELISA (Biosource International, Camarillo, CA).
RESULTS

*P. carinii* β-glucans induce degradation of cytosolic IKB-α in macrophages. Phosphorylation, ubiquitination, and degradation of I-κBα represent key events in the activation and translocation of NF-κB to the nucleus (14,17). We, therefore, sought to evaluate whether *P. carinii* β-glucans stimulate degradation of I-κBα during the course of macrophage activation with *P. carinii* β-glucans. RAW 264.7 macrophages were stimulated with *P. carinii* β-glucans and I-κBα levels evaluated by immunoblot analysis of cytosolic fractions (Figure 1). Stimulation of macrophages with *P. carinii* β-glucans induced degradation of I-κBα, which was evident following two hours of stimulation and appeared to be virtually absent in cytosolic extracts following four hours of incubation. To contrast these effects of *P. carinii* β-glucan to another potent stimulus of I-κBα degradation, we further compared the kinetics to those resulting from LPS-stimulation. In comparison, LPS induced more rapid degradation of I-κBα, evident as soon as 15 minutes of macrophage stimulation. This rapid degradation of cytosolic I-κBα following LPS stimulation is consistent with prior published observations (33) and illustrates that although *P. carinii* β-glucans and LPS both induce I-κBα degradation, they exhibit markedly different time courses, strongly suggesting use of different receptor-ligand interactions.

*P. carinii* β-glucans stimulate nuclear translocation of p65 NF-κB in macrophages. Degradation of cytosolic I-κBα enables NF-κB subunits to migrate to the nuclear compartment, where they promote gene transcription. We next investigated whether *P. carinii* β-glucans...
induce translocation of NF-κB components to nuclear compartments of stimulated macrophages. Since the p65 subunit of NF-κB has been demonstrated to exert critical activity in the transcription of many inflammatory genes (14, 17), we performed immunostaining to detect the nuclear translocation of the p65 subunit of NF-κB (Figure 2). While p65 NF-κB was localized predominantly in the cytoplasm of unstimulated RAW macrophages, stimulation with *P. carinii* β-glucans causes p65 NF-κB translocation to the nucleus in a time dependent manner. This response was detectable as early as 1 hour following stimulation and peaked between two and four hours of stimulation (Figure 2A). Nuclear translocation of p65 NF-κB began to decline following six hours of continuous stimulation with *P. carinii* β-glucan.

In contrast, parallel experiments conducted with LPS revealed significant p65 NF-κB translocation to the nucleus as rapidly as 15 to 30 minutes of stimulation. Subsequent time points showed decreasing levels of p65 NF-κB inside the nuclear compartment (Figure 2B). This redistribution of p65 NF-κB over time was evaluated by quantifying the nuclear/cytoplasmic ration of fluorescence intensity (Figure 2C). *P. carinii* cell wall β-glucans transiently induced p65 NF-κB nuclear translocation peaking between two to four hours (N/C ratios of 0.848 ± 0.050 and 0.826 ± 0.026, respectively), whereas LPS-induced p65 NF-κB translocation had an abrupt increase between 15 and 30 min (0.903 ± 0.023 and 0.884 ± 0.020, respectively) and rapidly declined by one hour (0.640 ± 0.030). Thus, consistent with our earlier observations on I-κBα degradation, *P. carinii* cell wall β-glucan stimulates NF-κB translocation in macrophages in a delayed fashion, but NF-κB activation is sustained for a much longer period of time, compared to that following LPS challenge.
Kinetics of NF-κB activation in RAW 264.7 macrophages stimulated with *P. carinii* β-glucan. To further understand *P. carinii* β-glucan activation of macrophages, DNA binding activity of NF-κB was examined in nuclear extracts of RAW 264.7 cells stimulated with *P. carinii* cell wall β-glucan by EMSA. An increase in nuclear NF-κB content occurred in nuclear extracts of macrophages stimulated with *P. carinii* cell wall β-glucans (Figure 3A). *P. carinii* β-glucan induced NF-κB binding was evident one hour after stimulation and appeared to decline by six hours, once again in marked contrast with LPS-induced nuclear NF-κB binding activity. LPS induced NF-κB binding was evident following ten minutes of stimulation, peaked between 15 and 30 min, and was largely reduced at later time points (Figure 3B and 3C). In contrast, *P. carinii* β-glucan induced NF-κB binding was slower in onset, and was not detected at these earlier time points (Figure 3C).

To next determine which components of NF-κB were present in the DNA-protein complexes, supershift and competition assays were performed (Figure 4). Supershift analysis demonstrated an upper band formed by a heterodimer of p65 and p50 subunits, and a lower band representing a homodimer of p50. Furthermore, oligonucleotide competition assays demonstrate that the DNA-protein complexes were formed specifically by interactions between the NF-κB probe and cognate nuclear proteins, since DNA binding of the radiolabeled NF-κB probe was fully inhibited by addition of cold NF-κB probe but not by equimolar concentrations of unlabeled AP-2 probe. Taken together, these data strongly indicate that *P. carinii* β-glucans induce macrophage degradation of IκB-α and nuclear translocation of p65 NF-κB. Furthermore, the kinetics of *P. carinii* β-glucan induced NF-κB activation are distinctly
different from those following LPS stimulation.

*P. carinii* β-glucan stimulated nuclear translocation of p65 NF-κB is inhibited by pyrrolidinedithiocarbamate. To begin to investigate whether pharmacological inhibitors could be potentially used to alter macrophage activation during challenge with *P. carinii* β-glucan, we studied whether PDTC, a specific and potent inhibitor of NF-κB (34, 35), would suppress activation of this transcription factor under these conditions. RAW 264.7 macrophages were preincubated with PDTC for 30 minutes, followed by four hours of stimulation with the *P. carinii* cell wall β-glucan isolate. Treatment with PDTC dramatically reduced p65 translocation to the nucleus upon stimulation with the *P. carinii* cell wall isolate (Figure 5A). RAW cell viability was not impaired by these concentrations of PDTC using the XTT viability assay.

To further determine whether PDTC suppression of NF-κB nuclear translocation was also associated with inhibition of inflammatory activation of macrophages, RAW cells were stimulated with *P. carinii* β-glucan in the presence or absence of PDTC (Figure 5B). Release of the pro-inflammatory cytokine TNFα was subsequently determined by ELISA. TNFα release following *P. carinii* β-glucan challenge was significantly suppressed by the NF-κB inhibitor in a concentration dependent fashion (P<0.05 comparing *P. carinii* β-glucan stimulated RAW cells in the presence versus absence of PDTC). RAW cell viability was not adversely affected by the agent. Taken together, these data indicate that *P. carinii* β-glucan induced translocation of NF-κB is associated with macrophage activation to release inflammatory mediators such as TNFα.

*P. carinii* β-glucan activation of alveolar macrophages is independent of TLR-4, but is
partially mediated through MyD88 dependent mechanisms. The striking similarities between LPS and fungal β-glucan-induced macrophage activation and cytokine release suggests parallel, though independent, mechanisms of inflammatory activation. The differential kinetics of I-κBα degradation and nuclear translocation of NF-κB strongly support that macrophage activation induced by \( P.\ carinii \) β-glucan isolates is unrelated to contaminating LPS. In addition, we performed exhaustive washes of our \( P.\ carinii \) cell wall isolate and confirmed the absence of contaminating endotoxin in the final \( P. carinii \) β-glucan preparations.

Toll receptors that interact with fungal β-glucans, have not yet been defined. To address the potential roles of TLR-4 in mediating β-glucan responses, we investigated the ability of \( P.\ carinii \) cell wall β-glucan and LPS to differentially activate TLR-4 deficient macrophages. Alveolar macrophages obtained from TLR-4 knockout mice were stimulated in parallel with either \( P.\ carinii \) β-glucan or LPS (Figure 6). As anticipated, LPS-induced TNF-α release in TLR-4 deficient alveolar macrophages was strikingly suppressed. For instance, LPS stimulation of TLR-4 -/- macrophages induced the release of only 5,829 ± 1,216 pg/ml of TNF-α compared to 27,043 ± 1,506 pg/ml of TNF-α released from wild type alveolar macrophages (P=0.0001). In contrast, stimulation of TLR-4 -/- macrophages with \( P. carinii \) β-glucan resulted in 33,627 ± 3,952 pg/ml of TNF-α, which was not significantly different compared to wild type macrophages. Similar data was obtained upon stimulation of alveolar macrophages from TLR4 deficient mice with glucan preparations of the phylogenetically related fungus \( S.\ cerevisiae \) (data not shown). Taken together, these data convincingly demonstrate that the \( P. carinii \) cell wall β-glucans activate alveolar macrophages through receptors systems distinct from
the classical LPS receptor TLR-4. In addition, these finding conclusively indicate that LPS contamination is not responsible for macrophage stimulation following challenge with *P. carinii* β-glucan.

Many Toll-mediated responses require activity of the Toll adaptor protein MyD88 (22). To further assess the activity of this family of innate immune receptors in mediating β-glucan responses, alveolar macrophages were obtained from MyD88 -/- deficient mice and challenged with *P. carinii* β-glucan (Figure 7). MyD88 -/- alveolar macrophages exhibited significantly reduced, though not completely suppressed, release of TNF-α compared to wild-type controls (Figure 6B). While wild type control macrophages stimulated with *P. carinii* β-glucan released 22,743 ± 669 pg/ml of TNF-α, MyD88 macrophages released only 13,026 ± 487 pg/ml (P=0.001). These finding indicate that macrophage activation in response to *P. carinii* β-glucan, is in part mediated through MyD88 dependent mechanisms.
DISCUSSION

Macrophages orchestrate innate immunity by phagocytosing invading pathogens by coordinating local inflammatory responses. Both particulate and soluble beta glucans in fungi initiate macrophage-driven immune responses. We recently established that the opportunistic fungal pathogen, *Pneumocystis carinii*, assembles a beta-glucan rich cell wall, which in addition to providing structural support for the organism, also strongly induces the release of TNF-α and MIP-2 from alveolar macrophages (3,6). *P. carinii* beta-glucans activate innate host defense responses through ligation of cognate glucan receptors on leukocytes (6,12). However, specific signaling pathways involved in such activation have not been previously characterized. Our current investigations reveal that beta-glucans components of the *P. carinii* cell wall strongly induce macrophage inflammatory activation through activation and nuclear translocation of NF-κB. We further demonstrate, that glucan driven inflammatory activation can be significantly suppressed by pyrrolidine dithiocarbamate inhibition of NF-κB activity. Finally, our investigations reveal that *P. carinii* beta-glucans stimulate macrophage activation through mechanisms distinct from those utilized by bacterial LPS. In particular, *P. carinii* beta-glucans activation of macrophage, occurs independently of the classical LPS receptor, TLR-4, but does appear to be partially mediated by the MyD88 toll receptor adaptor protein.

Unlike the adaptive immune system, which requires days to weeks for generation of an effective response to infection, the innate system provides immediate and crucial host defenses against invading pathogens (19). This innate surveillance system responds by recognizing a
restricted repertoire of highly conserved surface molecules representing indispensable structural components found in large groups of microorganisms. These structures (LPS, mannan, peptidoglycan) are referred to as pathogen-associated molecular patterns and are recognized by pattern recognition receptors present on many cell types. One family of receptors that exerts pivotal functions in innate immunity is the IL-1R/TLR family. Stimulation via the IL-1R/TLR family leads to initiation of signaling cascades that culminate in activation of NF-κB and mitogen activated protein kinases (36). Prototypic of such responses is TLR4, which serves as a critical receptor conferring host cell recognition of LPS found on Gram-negative bacteria. In addition, most, though not all, toll receptor mediated cellular responses involve interaction of cognate toll receptors with the MyD88 adaptor protein to effectively initiate cell signaling resulting in inflammatory activation (21,31,37).

The current study provides further support that cell wall β-glucans represent pattern recognition molecules triggering innate immune responses to pathogenic fungi such as *Pneumocystis carinii*. The current study indicates that β-glucans components of the *P. carinii* cell wall activate macrophage NF-κB to stimulate expression of TNFα, a central mediator of lung inflammation during *Pneumocystis* pneumonia (38-40). Our study further reveals that the *P. carinii* cell wall glucan activates macrophages via receptors distinct from the TLR-4 receptor mediating cell responses to LPS. However, we further demonstrate that the MyD88 toll adaptor protein is at least partially responsible for conferring macrophage response to *P. carinii* cell wall glucan. Recent studies further indicate that curdulan, a linear non-pathogenic cell wall carbohydrate, signals macrophage activation through a Toll like receptor pathway since
expression of a dominant negative mutant of MyD88 rendered macrophages refractory to curdulan stimulation (41). Taken together, these data strongly implicate Toll-mediated processes in glucan initiated macrophage activation.

Alveolar macrophages exert essential functions in host defense against Pneumocystis carinii, mediating uptake and degradation of the organisms (8). In addition to Pneumocystis clearance, macrophages also strongly initiate inflammatory responses in the lung following interactions with β-glucan components of the organism, including secretion of TNFα, IL-6, IL-8, MIP-2, eicosanoid metabolites, and reactive oxidant species (6,12,38,39,42-44). These exuberant host inflammatory responses, though necessary for clearance of infection, further promote lung injury resulting in respiratory failure and excessive morbidity. Therapeutic maneuvers that limit excessive inflammation are beneficial to patients with Pneumocystis pneumonia (45-48). These observations provide considerable initiative to fully understand the mechanisms and signaling pathways induced by P. carinii cell wall glucans, which trigger inflammatory activation of macrophages (8).

The delay in macrophage NF-κB activation following fungal β-glucan stimulation is rather unique compared to activation of other innate immune responses. This delay may be related to the necessity of glucan particle internalization, novel protein synthesis, and/or recruitment of other secondary receptors, to efficiently trigger cell activation. A previous study has shown that differing phagocytic mechanisms occur during ingestion of β-glucan through distinct binding sites of the CR3 β-glucan receptor, again, possibly contributing to a delay in
NF-κB activation (49). In addition, it is likely that several different glucan receptors, including CR3, toll receptors, and dectin-1 are present on the surface of inflammatory cells, exhibiting different affinities for various glucan isolates (50-52) (53) (54). Thus, the composition and conformation of the β-glucans in the fungal cell wall isolate, as well as the combination of glucan binding sites and receptors present on macrophages and other host cells will modify host responses to these fungal cell wall structures.

Effective NF-κB activation also likely requires optimal receptor recruitment, co-receptor interactions and/or engagement of other signaling intermediates. Many Toll-like receptors depend on homo or heterodimeric interactions with other Toll like family members to elicit optimal responses (55). For example, two members of the TLR family, TLR-2 and TLR-6, together coordinately mediate macrophage activation by Gram-positive bacteria and yeast cell wall (56). Additional studies suggest that glucan polymers may cross link spatially separated membrane receptors to induce immunomodulatory effects. Thus the length and structural complexity of any species β-glucan likely modulate both the type as well as strength of the elicited host responses (57,58).

In summary, our study demonstrates that the β-glucan rich *P. carinii* cell wall is a potent stimulant of macrophage inflammatory responses through activation of NF-κB. The mechanisms of macrophage NF-κB activation induced by *P. carinii* are distinctly different from those employed by LPS, with β-glucan exhibiting delayed but more persistent activation kinetics. Furthermore, *P. carinii* β-glucan induced TNFα release occurs through a TLR-4
independent pathway, but is significantly suppressed in the absence of the Toll adaptor protein MyD88, suggesting elements of Toll activity in these responses. These studies provide a basis for initial characterization of the specific immune signaling pathways involved in macrophage activation during the course of *P. carinii* pneumonia. Careful characterization of these pathways may suggest novel therapeutic strategies to attenuate host inflammation and the related mortality that accompany this infection.
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Figures

Figure 1. Stimulation of RAW 264.7 macrophages with *P. carinii* β-glucan induces cytosolic degradation of IκB-α. RAW macrophage cells were treated with *P. carinii* β-glucan (2.5 x 10^6 particles/ml) for the indicated times and degradation of IκB-α levels in cytoplasmic lysates were determined by immunoblot analysis. In parallel, cells were stimulated with LPS (0.1 µg/ml) and IκB-α levels in cytoplasmic lysates were also determined. Shown are representative blots of three experimental runs.

Figure 2. *P. carinii* β-glucans stimulate nuclear translocation of p65 NF-κB in RAW macrophages. A. Cells were treated with *P. carinii* β-glucan (PCBG; 2.5 x 10^6 particles/ml) for the indicated times and nuclear translocation of p65 NF-κB visualized by immunostaining with a specific antibody recognizing p65. Immunostaining was performed with FITC-labeled secondary antibody yielding fluorescence in the green range. For reference the nuclei are stained with DAPI (blue). B. Parallel experiments were performed on cells treated with 0.1 µg/ml of LPS. Nuclear translocation of p65 NF-κB was visualized by immunostaining with a specific antibody recognizing p65 and a Texas-red conjugated secondary antibody yielding fluorescence in the red range. C. Quantitative differences in fluorescence intensities between nuclei and cytoplasm were calculated and plotted from three experiments.

Figure 3. Time course of DNA binding activity of transcription factor NF-κB in the nuclei of *P. carinii* β-glucan and LPS stimulated RAW macrophages. A. RAW cells were treated with *P.
*P. carinii* β-glucan (2.5 x 10⁶ particles/ml) for the indicated times. Nuclear fractions were isolated and DNA binding activity of NF-κB determined using EMSA. **B.** In parallel, nuclear extracts were obtained from RAW macrophages stimulated with LPS (0.1 µg/ml) at the indicated times and DNA binding activity of NF-κB determined by EMSA. **C.** DNA binding activity of NF-κB from RAW cells treated with LPS (0.1 µg/ml) or *P. carinii* β-glucan (2.5 x 10⁶ particles/ml) for shorter periods of time was also analyzed by electromobility shift assay.

**Figure 4.** Competitive inhibition and supershift analysis of NF-κB DNA binding in RAW macrophages challenged with *P. carinii* β-glucan. Lane 1 - EMSA performed using nuclear fractions derived from unstimulated RAW cells (control). Lanes 2 through 6 represent EMSA performed with nuclear proteins derived from RAW macrophages stimulated with *P. carinii* β-glucan (2.5 x 10⁶ particles/ml) for 2 hours. Lane 2 - EMSA using radiolabeled NF-κB probe, but no competitive probe nor added antibody. Lane 3 - Addition of excess unlabelled NF-κB probe inhibits binding of radiolabeled NF-κB probe, confirming specificity of the binding interactions. Lanes 4 and 5. Supershift assays performed with antibodies recognizing the p65 and p50 subunit of NF-κB protein, respectively, confirm the presence of these subunits in the DNA protein complexes. Lane 6. Fifty-fold excess unlabeled AP-1 probe did not alter the electromobility shift assay, further confirming the specificity of these interactions.

**Figure 5.** PDTC inhibited nuclear translocation of p65 NF-κB and also TNFα release in RAW macrophages stimulated with *P. carinii* β-glucan. **A.** RAW cells were treated for 30 min with
the NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC; 10 µM) prior to stimulation with *P. carinii* β-glucan (PCBG, 2.5 x 10^6 particles/ml) for four hours. Nuclear translocation of p65 NF-κB was visualized by immunostaining with a specific antibody recognizing p65 and a Texas-red conjugated secondary antibody yielding fluorescence in the red range. Nuclei are stained blue with DAPI for reference. Immunofluorescence demonstrates suppressed nuclear translocation of p65 NF-κB in PDTC treated cells challenged with *P. carinii* β-glucan.

**B.** To further determine whether PDTC also inhibited inflammatory activation of macrophages, RAW cells were treated with PDTC at the indicated concentrations for 30 minutes prior to and throughout subsequent stimulation with *P. carinii* β-glucan (PCBG, 2.5 x 10^6 particles/ml) over eight hours. Release of TNFα into the medium was determined by ELISA. TNFα release was significantly suppressed by the NF-κB inhibitor PDTC. Shown are mean ± SEM. (* Denotes P<0.05 comparing *P. carinii* β-glucan stimulated RAWs in the presence and absence PDTC.)

**Figure 6. Alveolar macrophage TNFα response to *P. carinii* β-glucan is independent of TLR-4.** Alveolar macrophages harvested from TLR4 -/- and wild-type BALB/c control mice were treated with *P. carinii* β-glucan (2.5 x 10^6 particles/ml) or LPS (0.1 µg/ml) overnight, and TNFα levels measured in the media by ELISA. While macrophage response to LPS is dependent on TLR-4, no significant differences were observed between *P. carinii* β-glucan stimulation in macrophages from wild type and TLR-4 -/- mice (* Denotes P<0.05 comparing identical stimulation conditions in wild type and TLR-4 -/- macrophages).
Figure 7. Alveolar macrophage TNFα response to *P. carinii* β-glucan is partially mediated through the MyD88, Toll adaptor protein. Alveolar macrophages harvested from MyD88 -/- and wild-type C57BL/6 control mice were treated with *P. carinii* β-glucan overnight, and TNFα release into the medium measured by ELISA. MyD88 -/- exhibited significant, though partial, suppression of TNFα release in response to *P. carinii* β-glucan. (* Denotes P<0.05 comparing *P. carinii* β-glucan stimulation in wild type and MyD88 -/- macrophages).
REFERENCES


**Figure 1**

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IKK-βα ➔
Figure 2A

*P. carinii* β-Glucan Stimulated
RAW Cell p65 Translocation

Control

30 min

1 hr

2 hr

4 hr

6 hr
Figure 2B

LPS-Stimulated RAW Cell p65 Translocation

Control

15 min

30 min

1 h
Figure 2C

NF-κB Activation in RAW Cells Stimulated with Either *P. carinii* β-Glucan or LPS

Fluorescence (arbitrary units) vs. Time (Minutes)
Figure 3A

C  15' 30' 1h  2h  4h  6h

<- p65/p50
<- p50/p50
<- Non-specific
Figure 3B

C 15' 30' 1h 2h 4h

<- p65/p50
<- p50/p50
<- Non-specific
Figure 3C

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<-p65/p50
<-p50/p50
Figure 5A

PDTC Inhibits *P. carinii* β-Glucan Stimulated Nuclear Translocation of p65 NF-κB in RAW Cells

Control RAWs (No PCBG)  |  RAWs + PCBG (No PDTC)  |  RAWs + PCBG + PDTC
Figure 5B

PC Glucan Induced TNF-alpha secretion from RAW macrophages in the presence of PDTC
Figure 6

[Graph showing TNF-alpha levels in response to P. carinii β-Glucan and LPS for BALB c Wild Type and TLR-4 −/− mice.]
Pneumocystis carinii cell wall beta-glucans initiate macrophage inflammatory responses through NF-kB activation
Frances Lebron, Robert Vassallo, Vishwajeet Puri and Andrew H. Limper

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