**Pneumocystis carinii** Cell Wall β-Glucan Induces Release of Macrophage Inflammatory Protein-2 from Alveolar Epithelial Cells via a Lactosylceramide Mediated Mechanism

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Infiltration of the lungs with neutrophils promotes respiratory failure during severe *Pneumocystis carinii* (PC) pneumonia. Recent studies have shown that alveolar epithelial cells (AECs), in addition to promoting PC attachment, also participate in lung inflammation by the release of cytokines and chemokines. Herein, we demonstrate that a PC β-glucan rich cell wall isolate (PCBG) stimulates the release of macrophage inflammatory protein-2 (MIP-2) from isolated AEC’s through a lactosylceramide dependent mechanism. The results demonstrate that MIP-2 mRNA and protein production is significantly increased at both early and late time points after PCBG challenge. Although CD11b/CD18 (Mac-1, CR3) is the most widely studied β-glucan receptor, we demonstrate that CD11b/CD18 is not present on AECs. This study instead demonstrates that pre-incubation of AECs with an antibody directed against the membrane glycosphingolipid lactosylceramide (CDw17) results in a significant decrease in MIP-2 secretion. Preincubation of the anti-CDw17 antibody with solubilized lactosylceramide reverses this effect. Furthermore, incubation of AEC’s with inhibitors of glycosphingolipid biosynthesis, including N-butyldeoxyojirimycin (NB-DNJ) and D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol-HCl (PDMP), also results in a significant decrease in AEC MIP-2 production following challenge with PCBG. These data demonstrate that PC β-glucan induces significant production of MIP-2 from AEC’s and that CDw17 participates in the glucan-induced inflammatory signaling in lung epithelial cells during PC infection.
INTRODUCTION

*Pneumocystis carinii* (PC) pneumonia remains a life threatening opportunistic infection in immunocompromised hosts. Despite availability of effective medications, the mortality from PC pneumonia ranges between 15% and 40% (1). Neutrophilic lung inflammation promotes diffuse alveolar damage and contributes significantly to the respiratory failure, which is characteristic of severe PC pneumonia (2). The exact mechanisms by which PC induce this lung inflammation are not fully understood, though recent studies indicate that β-glucan components of the organism cell wall drive neutrophil accumulation during this pneumonia (3).

*P. carinii* has recently been classified as a fungal organism based on RNA homology and evidence that PC assembles a cell wall rich in β-glucans (4,5). Fungal β-glucans consisting of 1,3-linked β-D-glucopyranosyl residues with varying amounts of 1,6-linked β-D-pyranosyl side chains represent essential structural components of many fungal cell walls (6,7). Purified β-glucans from various fungal species strongly induce the production of proinflammatory cytokines and chemokines from inflammatory cells (8-10). Previous studies demonstrate that the PC cyst cell wall contains complex carbohydrates rich in β-glucans, chitin and a mannose-rich major surface glycoprotein complex termed gpA (5,7). We have recently purified particulate β-glucan from *P. carinii* and have demonstrated that alveolar macrophages produce tumor necrosis factor-alpha (TNFα) and macrophage inflammatory protein-2 (MIP-2) in response to this PC cell wall component (3,11).
Fungal glucans have been shown to bind several different receptors. In particular, the β2 integrin CD11b/CD18 (CR3, Mac-1) has been extensively studied as a major leukocyte membrane receptor for β-glucans (12,13). CD11b/CD18 possesses one or multiple lectin binding sites on the C-terminal domain of the CD11b/CD18 α subunit capable of interacting with complex carbohydrates (14). Additional studies using leukocytes have further demonstrated that the cell membrane glycosphingolipid, lactosylceramide (CDw17), also interacts with β-glucans (15). Interaction of glucans with CDw17 has also been reported to promote enhancement of the oxidative burst response and NF-kB activation of neutrophils (16).

Histological studies consistently identify *P. carinii* organisms closely associated with the alveolar epithelium in infected human and animal tissues. Furthermore, the binding of *P. carinii* to alveolar epithelial cells is believed to be essential for establishment of infection (17). However, accumulating evidence demonstrates that pulmonary alveolar epithelial cells not only function as a passive barrier mediating gas exchange, but also actively participate in the host immune response. Alveolar epithelial cells are capable of processing and presenting antigen to T lymphocytes (18,19). Furthermore, isolated alveolar epithelial cells produce and secrete various cytokines and chemokines including TNFα and MIP-2 when stimulated with bacterial components like lipopolysaccharide (LPS) or whole organisms such as *Mycobacterium tuberculosis* (20-23).

MIP-2 is of particular interest in the pathogenesis of *P. carinii* pneumonia since it
represents the rodent homologue of the human C-X-C chemokine IL-8 and is thus a potent stimulant of neutrophil accumulation and activation (24-26). Although chemokine expression is an important component of the inflammatory response against PC pneumonia, exuberant expression can cause deleterious effects on respiratory system function. Bronchoalveolar lavage studies in humans demonstrate that greater degrees of lung inflammation, as evidenced by increased neutrophil burdens, are associated with worse oxygenation and increased mortality during *P. carinii* pneumonia (2).

The current study was therefore undertaken to determine the extent to which *Pneumocystis carinii* cell wall β-glucans, induce the expression of MIP-2 from primary AEC’s. We further sought to determine whether the mechanisms of AEC activation by PC β-glucans was mediated through CD11b/CD18, or through the alternate lactosylceramide (CDw17) associated β-glucan receptor. Enhanced local production of MIP-2 by AEC’s may significantly promote neutrophilic lung inflammation that characterizes severe *Pneumocystis carinii* pneumonia and contributes to respiratory failure in this infection.
MATERIALS AND METHODS

Materials. Unless otherwise noted, general reagents were from Sigma Chemical Co. (St. Louis, MO). *P. carinii* f. sp. (formae specialis) *carinii* was originally obtained through the American Type Culture Collection (ATCC) and maintained in our immunosuppressed rat colony.

The following antibodies were used: Anti-CD11b (M-19, Santa Cruz Inc., Santa Cruz, CA.), FITC anti-CD45 (OX-1, Pharmingen, San Diego, CA.), Anti-CDw17 (Huly-M13, Ancell, Bayport, MN), Anti-asialoGM1 (Matreya, State College, PA.). We recently described the purification and characterization of a β-glucan rich cell wall isolate from *P. carinii* (3). This isolate was found to contain 95.7% carbohydrate and 4.3% protein by weight using the orcinol-sulfuric acid method and BCA protein determinations, respectively. A sample of the *P. carinii* cell wall isolate was largely, though not completely, hydrolyzed with 2M HCl thereby releasing 82% of its content as D-glucose measured by the glucose oxidase method. Thus, the majority of this material represents *P. carinii* derived glucose polymer, namely glucans (3). This material was previously reported to be active in stimulating alveolar macrophages to release TNFα and was markedly degraded by β-1,3-glucanases, losing most of its stimulatory activity following digestion (3). The β-glucan rich *P. carinii* cell wall isolate was exhaustively washed with SDS (1%) and polymyxin (10 µg/ml) to remove any contaminating endotoxin. The final wash was assayed for endotoxin using a modified Limulus ameboocyte lysate assay (Whittaker M.A. Bioproducts, Walkersville, MD) and consistently found to contain <0.125 U/ml of endotoxin (3).
**Primary Alveolar Epithelial Cell Isolation.** Rat alveolar epithelial cells were isolated according to the method of Dobbs et al. (27). Briefly, Sprague-Dawley rats (~250g) were anesthetized by an intraperitoneal injection of pentobarbital (100 mg/kg) and exsanguinated by transection of the inferior vena cava. Blood was evacuated from the pulmonary vasculature by perfusion with normal saline. The trachea was isolated and the lungs lavaged multiple times to remove alveolar macrophages. AEC’s were separated from the basement membrane by filling and incubating the lungs with porcine elastase (1500 units/animal) for 20 minutes in situ. Next, the lungs were minced, filtered, and centrifuged (130 x g). The collected cells were resuspended in DMEM and incubated for one additional hour on petri dishes coated with rat IgG to remove macrophages by Fc-mediated adherence. The non-adherent cells were collected, centrifuged (400 x g for 10 min), and resuspended in DMEM with 10% bovine calf serum, penicillin (50,000 U/L) and streptomycin (50 mg/L) solution. Cells were counted using a standard hemocytometer.

Freshly isolated alveolar epithelial cells (3x10^5 cells/well) were plated on 96 well tissue culture plates, and incubated over 48 hrs (37°C, 5% CO_2), with the media being changed after the initial 24 hours. A sample of cells was also stained with FITC anti-CD45 (OX-1, Pharmingen) and viewed under fluorescence, to ensure minimal contamination by macrophages (<5%). These AEC monolayers derived after 48 hours of culture were used to study epithelial cell MIP-2 release following β-glucan stimulation.

**MIP-2 Protein Quantification.** To characterize AEC responses to the PC β-glucan-rich Hahn/Limper-8
cell wall fraction, varying concentrations (1-10 x 10^6 particles/ml) of the PCBG isolate were used to stimulate the alveolar epithelial cell monolayers. In preliminary studies, a concentration of (5x10^6 particles/ml) was found to cause optimal cell stimulation. AEC’s were then stimulated and supernatants were collected at 0, 2, 6, 12, 16 or 24 hrs. A MIP-2 sandwich ELISA (Biosource, Camarillo, CA.) was used to determine MIP-2 protein concentrations released into the media at the different time points. To further determine the relative potency of MIP-2 release from AEC’s compared to alveolar macrophages (AMS), and to exclude the possibility that residual macrophages in the AEC preparations significantly contributed to the observed MIP-2 release, identical numbers of AEC’s and alveolar macrophages (3 x 10^5 cells/well), as well as the maximal number of residual alveolar macrophages in the AEC isolates (5% AMS, equal to 1.5 x 10^4 cells) were stimulated with PCBG (5 x 10^6 particles/ml) for 24 hours. MIP-2 release into the medium was again determined by ELISA.

**MIP-2 mRNA Determination.** To further characterize AEC expression of MIP-2, ribonuclease protection assays (RPA) were performed to evaluate steady-state MIP-2 mRNA levels following stimulation with the *P. carinii* β-glucan. Rat alveolar epithelial cell monolayers (5 x 10^6 cells/well) were stimulated with *P. carinii* β-glucan (5 x 10^6 particles/well) for 0, 2, 6, and 24 hours and MIP-2 RNA was measured by ribonuclease protection assay. To confirm our findings in primary AEC, and to further exclude the possibility that MIP-2 was derived from residual alveolar macrophages in the AEC preparations, MLE-12 cells (ATCC, Rockville, MD), Hahn/Limper-9.
a transformed alveolar type II cell line, were similarly cultured with identical concentrations of
*P. carinii* β-glucan at parallel time points. Total RNA was isolated at the end of each time point
using the RNeasy Mini Kit (Qiagen Inc., Valencia, CA). Ribonuclease protection assays was
then performed according to the manufacturers instructions (Pharmingen Inc., San Diego, CA).
Briefly, 15 µg of RNA was precipitated using ammonium acetate and ethanol. A custom
cytokine template set and the T7 polymerase were used to transcribe \([P^{32}]\)-radiolabeled,
antisense probes for rat MIP-2, MCP-1, IL-1α, TNFα, TNFβ, IL-3, IL-4, IL-5, IL-10, IL-2,
IFNγ, L32, and GAPDH. The radioprobes were hybridized in excess of the target RNA. At the
end of the hybridization, free probe and other single-stranded RNA were digested with RNase.
The RNase protected probes were purified using chloroform/isoamyl alcohol/phenol and
precipitated with ethanol. The probes were then resuspended in Laemmli loading buffer,
separated on 6% polyacrylamide gels, dried, and exposed to X-ray film.

**In Vivo Determination of MIP-2 mRNA levels.** Intratracheal instillation of PC β-glucan was used to
evaluate the *in vivo* expression of MIP-2 by AEC’s. Sprague-Dawley rats were briefly
anesthetized, the trachea isolated through a neck incision and 0.5 ml of *Pneumocystis carinii* β-
glucan rich cell wall isolate (2x10^7 particles/ml) injected into the lungs. After 24 hours, alveolar
epithelial cells were isolated from the inoculated rats as described above. A sample of cells was
also stained with FITC anti-CD45 (OX-1, Pharmingen) and viewed under fluorescence, to
ensure minimal contamination by macrophages (<5%). RNA was immediately extracted from
the freshly isolated AECs and RPA performed to determine MIP-2 mRNA levels.

Hahn/Limper-10
Immunoprecipitation and Immunoblot Analysis for CD11b. Since the integrin CD11b/CD18 represents a major well-characterized β-glucan receptor, we evaluated whether it was present on cultured AECs. AEC’s (5 x 10^6) were cultured over 48 hours. Rat macrophages (2 x 10^6) were also isolated from the same animals to serve as a reference source for the CD11b integrin. The cells were extracted with octyl-thioglucoside (100 mM) containing a protease inhibitor cocktail (Complete Mini, Roche, Mannheim, Germany). Lysates were precleared with 1 μg of goat IgG and 20 μl of Protein G-Sepharose for 30 minutes. Two μg of Anti-CD11b (M-19, Santa Cruz) or goat IgG was added to lysates containing 200 μg of cellular protein, and incubated for two hours. Twenty μl of Protein-G Sepharose was then added to the lysates and incubated an additional hour. Immunoprecipitates were collected by centrifugation 1,000 x g for 5 minutes, washed and resuspended in Laemmli buffer. The immunoprecipitated products were separated over a 10% Tris-HCL SDS-PAGE gel and CD11b detected by immunoblotting with anti-CD11b using streptavidin-biotinylated alkaline phosphatase complex detection.

Effect of anti-CD11b, anti-asialoGM1, and anti-CDw17 on AEC MIP-2 Release. Recent studies indicate that lactosylceramide (CDw17) may function as an alternate β-glucan receptor mediating cellular activation (16). To test this, freshly isolated AECs (3x10^5) were plated on 96 well plates and cultured for 48 hr. Cells were next pre-incubated for 20 minutes with anti-CD11b, anti-CDw17, anti-asialoGM1 or isotype control mouse IgM (Sigma, St. Louis, MO), and stimulated with the PCBG (5x10^6 particles/ml) for 16 hours in the presence of
these antibodies at the indicated concentrations. In an identical fashion, AEC’s were also cultured with LPS (1.0 µg/ml), an independent potent stimulant of epithelial cells. Supernatants were collected after 16 hours and MIP-2 concentrations determined by ELISA. Cell viability was confirmed using the Cell Proliferation Kit II (Roche, Mannheim, Germany). This assay measures the conversion of sodium-3’-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonyl acid hydrate (XTT) to a formazan dye through electron-coupling in metabolically active mitochondria using the coupling reagent N-methyl dibenzopyrazine methyl sulfate. Only metabolically active cells are capable of mediating this reaction, which is detected by absorbance of the dye at 450-500 nm.

To further confirm specificity of the antibody, in parallel experiments, anti-CDw17 was also preincubated with solubilized lactosylceramide (LacCer, 1 mg/ml) for 20 minutes to neutralize the antibody prior to addition to the cells. Immunohistochemistry (IHC) was performed to verify that both the anti-CDw17 and the anti-asialoGM1 antibodies specifically interacted with the cultured AEC’s. Immunohistochemistry was also performed on AEC monolayers which had been digested with endoglycosidase Hf (New England Biolabs, 1000U/ml, 24 hours), to remove sugar modifications of the cellular surfaces. Finally, to further exclude steric hindrance effects of the bulky IgM anti-CDw17 antibody, in additional experiments, the IgM was digested into IgG and rIgG fragments prior to use (ImmunoPure IgM Fragmentation Kit; Pierce Inc., Rockford, IL).

Effect of Lactosylceramide Synthesis Inhibition on Glucan-Induced Release of MIP-2

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by AEC’s. We further investigated whether reduction of AEC membrane lactosylceramide would reduce MIP-2 production following β-glucan challenge. AEC’s (3x10^5) were cultured in DMEM/F12 containing lipid free FCS (Intracel, Issaquah, WA.), in the presence of the glycosphingolipid biosynthesis inhibitors, N-butyldeoxynojirimycin (NB-DNJ; 200 µM, 400 µM) or D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol-HCl (PDMP; 20 µM) (28-30). Cells were incubated with NB-DNJ, PDMP, or lipid free media alone for 72 hours and subsequently stimulated with PC β-glucan (5x10^6 particles/ml) for 16 hours. MIP-2 release was determined by ELISA. Toxicity to the AEC’s induced by either NB-DNJ and PDMP was again determined using the XTT Viability Assay (Roche). To further study the effect of an independent stimulus, AECs were cultured with LPS (1.0 µg/ml), and MIP 2 release determined in the presence or absence of NB-DNJ (400 µM)

**Statistical Analysis.** All data are expressed as the mean ± SEM. Differences between groups were determined using two-tailed Student’s t test. Statistical testing was performed using SPSS software program, with statistical differences considered significant if P<0.05.
RESULTS

*P. carinii* β-glucan Cell Wall Isolate Induces the Release of MIP-2 from Alveolar Epithelial Cells. AEC’s responded to stimulation with *P. carinii* β-glucan by release of the chemokine MIP-2. After six hours of stimulation MIP-2 production was significantly increased with concentrations of PCBG as low as 1x10^6 particles/ml, which caused 165 ± 95 fold more MIP-2 release compared with unstimulated controls (P=0.003). The response of AEC’s occurred fairly rapidly after PCBG stimulation (Figure 1A). MIP-2 levels after PC β-glucan (5x10^4 particles/ml) stimulation were significantly increased as early as 2 hours post stimulation. MIP-2 secretion was further enhanced at 6 through 24 hours with maximal accumulation of MIP-2 in the media observed at 16 and 24 hours. Thus, AEC’s respond to PCBG stimulation by release of MIP-2 in a graded time-dependent fashion.

To further determine the relative potency of the AEC response to PC β-glucan compared to alveolar macrophages, equal numbers of AEC’s and macrophages (3x10^5 cells/well) were cultured with PC β-glucan for 24 hours under identical conditions and the level of MIP-2 in the medium determined by ELISA (Figure 1B). Under these conditions, the AEC’s release 7510 ± 376 pg/ml of MIP-2. In contrast, equal numbers of macrophages released only 2911 ± 532 pg/ml of MIP-2 (P<0.01). Thus, alveolar epithelial cells are a potent source of MIP-2 following challenge with this *P. carinii* cell wall component. To further exclude the possibility that residual macrophages present in the AEC isolates were responsible for the MIP-2 release in response to PC β-glucan, we determined that the AEC preparations maximally contained 5% residual

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macrophages at the time of isolation. This number of macrophages (1.5x10^4 cells/well), were also concurrently cultured with PC β-glucan, and found to release only 89 ± 117 pg/ml of MIP-2 (P<0.001 compared to the AEC isolates challenged with PCBG) (Figure 1B). Thus, it is unlikely that residual macrophages contributed significantly to the observed MIP-2 release in the AEC preparations.

**P. carinii β-glucan Stimulates Enhanced Steady-State MIP-2 mRNA in Alveolar Epithelial Cells.** Next, ribonuclease protection assays (RPA’s) were performed to determine whether glucan stimulation similarly increased MIP-2 RNA expression in primary alveolar epithelial cells (Figure 2A). Steady state MIP-2 mRNA was significantly increased following two hours of *P. carinii* β-glucan stimulation compared to unstimulated control. A doublet hybridization pattern was observed for MIP-2. Further increases in MIP-2 mRNA were also noted after 6 and 24 hours of stimulation. The total achieved level of MIP-2 RNA expression in AEC’s was comparable and exceeded that elicited by six hours of challenge with TNFα (100 ng/ml), an established stimulant of MIP-2 expression. RNA loading was verified by hybridization to the constitutively expressed L32 and GAPDH genes. In addition, consistent with our prior studies, RPA on primary AECs further demonstrated an increased level of TNFα and MCP-1 mRNA at both 6 and 24 hours after stimulation with *P. carinii* β-glucan (3,11).

Although the AEC preparations consistently contained >95% epithelial cells, with few remaining macrophages, we further confirmed that *P. carinii* β-glucan increase MIP-2 expression in MLE-12 cells, an immortalized alveolar epithelial cell line, free of any

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macrophage contamination (Figure 2B). RPA using MLE-12 cells demonstrated that MIP-2 mRNA was increased 2 and 4 hours after PC β-glucan stimulation compared to controls. Interestingly, an increase in the murine chemokine TCA3 mRNA was also observed following 2 and 4 hours of PCBG stimulation in MLE12 cells. The MLE12 cell line exhibited MIP-2 expression following challenge with PCBG, providing further confirmation that alveolar epithelial cells can directly respond to PCBG with release of the MIP-2 chemokine in the complete absence of macrophages.

*P. carinii* β-glucan Stimulates MIP-2 mRNA Expression in Alveolar Epithelial Cells *in Vivo.* We have previously reported that PC β-glucan induces substantial neutrophilic lung inflammation and enhanced lung MIP-2 levels. Thus, we next sought to determine whether PC β-glucan similarly enhanced MIP-2 gene expression in alveolar epithelial cells following intratracheal instillation *in vivo* (0.5 ml containing $1 \times 10^7$ PCBG particles). Twenty-four hours following challenge, alveolar epithelial cells were isolated from the rats, and MIP-2 expression analyzed by RPA (Figure 3). The 24-hour time point was chosen as a steady state time point at which neutrophil recruitment and cytokine expression are well established following PC β-glucan challenge in our previous studies (3). MIP-2 mRNA expression was enhanced in the PC β-glucan stimulated animals compared with control animals. Minimal basal MIP-2 RNA expression was found in the unstimulated controls. As described above, RNA was immediately isolated after the alveolar cell isolation procedure and the AEC’s were not maintained in culture. Again, CD45 fluorescence immunostaining of the AEC preparations consistently confirmed less
than 5% contamination with alveolar macrophages. These data demonstrate that AEC’s express MIP-2 mRNA following challenges in the intact host. Under such in vivo conditions epithelial cells may be acting in direct responses to PC β-glucan, but MIP-2 responses may also be related to macrophage activity, including the release of factors such as TNFα, which are known to stimulate AECs.

**Alveolar Epithelial Cells Lack the CD11b/CD18 β-Glucan Receptor.** The integrin CD11b/CD18 is present on leukocytes and initiates cell activation in response to β-glucans (12-14). Although no prior reports of CD11b/CD18’s presence on AEC’s could be identified, we sought to independently confirm that this β-glucan receptor was absent from primary AEC cultures (Figure 4). Sequential immunoprecipitation and immunoblot analysis clearly demonstrated that CD11b, while present on alveolar macrophages, was lacking from the alveolar epithelial cells, indicating that an alternate receptor must be mediating MIP-2 activation in response to PC β-glucan. This sequential immunoprecipitation and immunoblot analysis further verified that viable alveolar macrophages represented only a negligible component of the primary AEC isolates.

**Antibodies to Lactosylceramide (CDw17) Strongly Inhibit MIP-2 Release by AECs Challenged with P. carinii β-Glucan.** As anticipated from the preceding experiments, anti-CD11b did not significantly inhibit MIP-2 production following stimulation with PC β-glucan, further indicating that AECs must utilize an alternate β-glucan receptor (Figure 5A).
Lactosylceramide has recently been shown to function as such an alternate β-glucan receptor (16). In contrast to anti-CD11b, incubation of AECs with anti-CDw17, a mouse monoclonal IgM antibody, markedly suppressed MIP-2 production as compared to isotype matched IgM non-immune control (Figure 5A). The maximum degree of inhibition was found with an antibody concentration of 200 µg/ml. In contrast, anti-asialo-GM1, an antibody recognizing another glycosphingolipid found in mammalian membranes, had no discernable effect on MIP-2 release. Immunohistochemical staining confirmed strong binding of both the anti-CDw17 and the anti-asialo-GM1 antibodies to AEC cultures. Staining for both CDw17 and asialo-GM1 was eliminated following digestion of the cells with endoglycosidase Hf (data not shown).

To further verify the specificity of suppression induced by anti-CDw17 on MIP-2 release by AECs in response to PCBG, additional experiments were performed to assess the ability of AECs to release MIP-2 following stimulation with LPS (1.0 µg/ml) in the presence of this antibody (Figure 5B). LPS stimulates cells using receptor systems distinct from those known to interact with β-glucans (3). Notably, AECs were well stimulated to release MIP-2 following challenge with LPS, and this response was not altered by the presence of anti-CDw17. Thus, antibodies to lactosylceramide (CDw17) specifically inhibit the responses of AEC’s to PC β-glucan, yet the cells remain viable and able to respond normally to an alternative stimulant such as LPS.

The ability of the anti-CDw17 antibody was significantly, though partially, reversed by
pre-incubation of the antibody with solubilized lactosylceramide before being added to the AEC’s (Figure 6). To exclude the possibility that the inhibitory effects were solely related to steric hindrance imparted by the bulky IgM anti-CDw17 binding to the membrane, the anti-CDw17 (IgM) was digested into IgG and rIgG fragments. Again, when AEC’s were incubated with fragmented anti-CDw17 (IgG and rIgG), significant decreases in MIP-2 still occurred following β-glucan stimulation, comparable to the effects of the whole IgM anti-CDw17 antibody (Figure 6). Taken together, these data strongly implicate that lactosylceramide (CDw17) participates in the membrane complex mediating AEC’s activation to release MIP-2 following PCBG stimulation.

Inhibition of Lactosylceramide Production in AEC’s Decreased MIP-2 Production Following Stimulation with PC β-Glucan. A number of agents have been produced which inhibit the synthesis of glycosphinogolipids (28-30). Such compounds may represent novel means to alter P. carinii-induced lung inflammation. To test this, cellular generation of lactosylceramide (CDw17) in AEC’s was inhibited with NB-DNJ and PDMP, two potent inhibitors of glycosphingolipid synthesis (Figure 7A). After 72 hr incubation with NB-DNJ (400 µM), there was significantly reduced MIP-2 release following β-glucan stimulation compared to control cells incubated in lipid-free media alone. Similarly, incubation of AEC’s with PDMP (20 µM) also resulted in significantly decreased levels of MIP-2 production from AEC’s following P. carinii glucan challenge. Neither NB-DNJ and PDMP altered cellular viability as measured by the XTT viability assay.

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Additional experiments we performed to assess the ability of AEC’s to release MIP-2 following stimulation with a potent alternative agent, namely LPS (1.0 µg/ml), in the presence of the glycosphinogolipid synthesis inhibitor (Figure 7B). Again, AECs were potently stimulated to release MIP-2 following stimulation with LPS, and this release was not significantly altered by the presence of NB-DNJ. Thus, inhibition of glycosphinogolipid synthesis specifically inhibits AEC release of MIP-2 in response to PCBG, yet the cells remain fully viable and are able to respond normally to an alternative stimulant such as LPS, which acts through other receptor pathways.
DISCUSSION

Despite effective antimicrobial agents, *Pneumocystis carinii* pneumonia continues to inflict a high rate of respiratory failure and death. Severe PC pneumonia is characterized by intense neutrophilic lung inflammation leading to impaired gas exchange, diffuse alveolar damage, and respiratory failure. In fact, neutrophil infiltration in the lungs of patients with PC pneumonia is a stronger predictor of respiratory failure and death than the organism burden itself (2). Additional investigations indicate that *P. carinii* cell wall β-glucans strongly stimulate neutrophilic lung inflammation (3,11). Non-specific anti-inflammatory therapies, such as adjunctive corticosteroids, have significantly decreased mortality during severe PC pneumonia (31). Thus, a better understanding of glucan-induced inflammation may provide novel means by which lung inflammation can be selectively modulated during this infection.

It is well established that alveolar macrophages produce and secrete cytokines and chemokines such as TNFα and MIP-2, which are potent inducers of neutrophil migration and activation (3). It is, however, becoming more evident that alveolar epithelial cells are also active participants in the host’s inflammatory response (20,21,32). For instance, Standiford reported that A549 alveolar epithelial cells produce significant quantities of IL-8, the human functional homologue of MIP-2, when stimulated with TNFα (32). However, A549s did not express IL-8 in response to LPS alone, and these authors postulated the existence of a cytokine network model whereby alveolar epithelial cells are stimulated by activated alveolar macrophages (32).
contrast, primary alveolar epithelial cell cultures have been shown to produce both TNFα and MIP-2 in direct response to LPS (14). The current study is the first to demonstrate that the β-glucan cell wall fraction from *Pneumocystis carinii* can induce production of significant quantities of the chemokine, MIP-2, from alveolar epithelial cells, further implicating the alveolar epithelium as an important participant in host inflammation during lung infection.

It is important to note that strict precautions were taken to minimize alveolar macrophage contamination in the AEC isolates used in these studies. Anti-CD45 immunofluorescence consistently demonstrated fewer than 5% macrophages in these preparations, and negligible CD11b, a prominent macrophage marker, was detected by sequential immunoprecipitation and immunoblotting. Our data further demonstrate that, on a cell-by-cell basis, AEC’s secrete greater amounts of MIP-2 than alveolar macrophages. In addition, assuming a 5% contamination of the AEC with macrophages, that number of macrophages would have only generated minimal MIP-2 (89 pg/ml) in these cultures (3,11). Thus, it is highly unlikely that the substantial levels of MIP-2 produced by *P. carinii* β-glucan stimulation of the AEC cultures were related to the few residual macrophages in these isolates. Furthermore, the murine alveolar epithelial cell line, MLE-12, which is free of any other cell population also demonstrated a significant increase in MIP-2 mRNA after PC β-glucan stimulation. In light of the greater numbers of alveolar epithelial cells in the lung contrasted to macrophages and other cells, the direct response of AECs to β-glucan components of the PC cell wall likely represents a significant source of MIP-2 during this infection. It does, however, remain possible that macrophages may also release factors that augment epithelial cell release of chemokines such as MIP-2 during *in vivo* challenge.

Hahn/Limper-22
Although MIP-2 is an important chemokine involved in lung inflammation, it is only one of a number of neutrophil directed cytokines. Our RPA analysis indicates that AEC also increase expression of TNFα and MCP-1 following PCBG stimulation. Prior studies by our group and others have documented that TNFα is essential for optimal clearance of *P. carinii* infection (3,11,33-35). Additional studies indicate that MCP-1 is also significantly expressed in rodent lungs during *P. carinii* infection and by A549 cells stimulated with *P. carinii* major surface glycoprotein (36,37). We instead have focused on the stimulatory effects of *P. carinii* β-glucan because our prior studies have indicated this component to principally mediate lung inflammatory responses during *P. carinii* infection (3,11). In addition to observing enhanced expression of MIP-2, we also observed that TCA3 mRNA expression was increased after PC β-glucan stimulation in MLE12 cells. TCA3 is an activation specific cytokine product previously described to be produced by T cells and Mast cells and to function as a potent chemotactic agent for neutrophils and macrophages (38,39). We believe this is the first demonstration that epithelial cells express TCA3. These studies strongly implicate lung epithelial cells as an integral component in the recognition and response of the host to *P. carinii*, with epithelial cell-derived MIP-2 serving to promote neutrophil recruitment during infection. Moreover, since β-glucans are common throughout the pathogenic fungi, this innate response may represent a generalized response of the epithelium to fungal infection.

Several potential β-glucan receptors have been reported in the literature. The most widely studied is the β2 integrin CD11b/CD18, which possesses a lectin-binding site on CD11b...
that has specificity for β-glucan. This receptor is cross-linked by high molecular weight soluble and particulate β-glucans leading to the activation of phagocytic cells (40,41). The current study demonstrates, however, that CD11b/CD18 is not present on epithelial cells to any significant extent and does not function as a glucan receptor mediating AEC activation. Very recent studies have also documented an additional β-glucan receptor, namely dectin-1, which is largely present on macrophages and dendritic cells (42,43). Its presence in epithelial and other cells has not yet been established. Instead, our study instead implicates lactosylceramide as an epithelial cell receptor participating in AEC activation to release MIP-2 in response to \textit{P. carinii} β-glucan components.

The glycosphingolipid, lactosylceramide (LacCer), is widely distributed in the plasma membranes of many different cell types including alveolar epithelial cells. LacCer has been shown to bind several different bacteria and yeast (41). Recently, LacCer has been demonstrated to bind PGG-glucan, a purified β-glucan derived from the cell wall of \textit{Saccharomyces cerevisiae} (15). The binding of PGG-glucan to LacCer activates a NF-κB-like nuclear transcription factor in human polymorphonuclear cells (16). Herein, we demonstrate that antibodies to cell membrane LacCer (CDw17) and inhibitors of LacCer synthesis significantly attenuate the secretion of MIP-2 after PC β-glucan challenge in alveolar epithelial cells. The glycosphingolipid biosynthesis inhibitors N-butyldeoxynojirimycin (NB-DNJ) inhibits the first step in glycosphingolipid biosynthesis, where glucose is transferred to ceramide. Culture of cells with NB-DNJ causes a depletion of glycosphingolipids including LacCer (28,29). In contrast,
D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) is an inhibitor of glycosyl transferase (29). Incubation of AEC’s with either NB-DNJ or PDMP causes a significant decrease in MIP-2 release after stimulation with PC glucan. Such compounds might represent a novel means to similarly modulate *P. carinii* induced inflammation *in vivo*.

How CDw17 transmits its signal has yet to be elucidated. Accumulating evidence indicates that glycosphingolipids are concentrated in microdomains on the plasma membrane surface (44,45). These microdomains or “rafts” are thought to represent platforms where signaling molecules are concentrated. Interestingly, antibodies to asialoGM1, another glycosphingolipid on AEC’s and a known *Pseudomonas* pilin receptor, did not result in any attenuation of MIP-2 production. It might be postulated that ligation of β-glucan in microdomains may result in concerted uptake of β-glucan particles and recruitment and activation of signaling kinases within these microdomains.

In summary, our study demonstrates that β-glucan components of the *Pneumocystis carinii* cell wall are able to stimulate alveolar epithelial cells to produce MIP-2. These investigations further demonstrate that the membrane glycosphingolipid lactosylceramide participates in the receptor complex mediating PC β-glucan activation of AEC’s. MIP-2 produced by AEC’s and macrophages significantly promotes neutrophilic lung inflammation during severe *Pneumocystis* pneumonia. Emerging strategies to attenuate glucan mediated inflammatory signaling, including inhibition of lactosylceramide-based signaling, may hold
therapeutic benefit to reduce lung injury during *P. carinii* pneumonia.
AKNOWLEDGEMENTS

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FOOTNOTES

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2 Abbreviations used: AEC, alveolar epithelial cell; MIP-2, macrophage inflammatory protein-2; TNF, Tumor necrosis factor α; PC, Pneumocystis carinii; PCBG, Pneumocystis carinii β-glucan-rich cell wall fraction; RPA, ribonuclease protection assay; MCP-1, monocyte chemoattractant protein-1; GAPDH, glyceraldehyde-3-Phosphate dehydrogenase; TCA3, T cell activation-3 cytokine; RANTES, regulated on activation normal T-cell expressed chemokine.
FIGURE LEGENDS

Figure 1. MIP-2 production from alveolar epithelial cells following stimulation with *Pneumocystis carinii* β-glucan cell wall isolate. A. Isolated AECs (3 x 10^5 cells/well in 200 µl) were stimulated with PCBG (5 x 10^6 particles/ml) for 0 hours (control), and 2, 6, 12, 16, or 24 hours, and MIP-2 release into the medium determined by ELISA. Data points are expressed as mean ± SEM (* Denotes P<0.05 compared to control). B. To assess the relative potency of MIP-2 release from AEC’s compared to alveolar macrophages (AMS), and to exclude the possibility that residual macrophages in the AEC preparations significantly contributed to the observed MIP-2 release, identical numbers of AEC’s and alveolar macrophages (3 x 10^5 cells/well), as well as the maximal number of residual alveolar macrophages in the AEC isolates (equal to 1.5 x 10^4) were stimulated with PCBG (5 x 10^6 particles/ml) for 24 hours. MIP-2 release into the medium was determined by ELISA. (* Denotes P<0.01 compared to 3 x 10^5 AEC’s).

Figure 2. MIP-2 RNA expression in lung epithelial cells after stimulation with *Pneumocystis carinii* β-glucan. A. Ribonuclease protection assay of primary AEC cultures (5 x 10^6 cells/well in 3 ml) were performed following stimulation with PC β-glucan rich cell wall isolate (5 x 10^6 particles/well) for 2, 6, or 24 hours. Control (“C”) denotes unstimulated cultured AEC’s. In
parallel cultures, AEC’s were also cultured with TNFα (100 ng/ml), a known stimulant of MIP-2 expression for six hours. B. In a parallel fashion, the MLE-12 murine alveolar epithelial cell line was assessed for chemokine RNA expression following PCBG challenge for 2 or 4 hours compared to unstimulated control AECs. Again, AECs were also stimulated with TNFα (100 ng/ml), for 4 hours. RNA loading was determined by assessing GAPDH and L32 expression.

**Figure 3.** MIP-2 mRNA expression in alveolar epithelial cells isolated from mice challenged intratracheally with PC β-glucan cell wall isolate. Twenty-four hours after intratracheal injection, lungs were removed and alveolar epithelial cells immediately isolated. MIP-2 mRNA expression was measured using RPA. Shown is a representative blot of three separate experiments. The first lane contains control unstimulated mouse lung RNA, the second lane contains +PCBG stimulated mouse lung RNA.

**Figure 4.** Alveolar epithelial cells lack the CD11b/CD18 β-glucan receptor. Primary alveolar epithelial cells and alveolar macrophages were isolated, membrane proteins extracted, and immunoprecipitated with anti-CD11b antibodies. The precipitated products were separated by SDS-PAGE, transferred and analyzed with immunoblot using an antibody recognizing the CD-11b integrin component receptor.

**Figure 5.** Effect of antibodies to lactosylceramide on MIP-2 release by alveolar epithelial cells incubated with *P. carinii* β-glucan. A. Isolated AECs were incubated with media alone,
non-immune mouse IgM, anti-CDw17, anti-CD11b, or anti-asialoGM1 (200 µg/ml each) for 30 minutes before and throughout a subsequent 16 hour challenge with the PC β-glucan rich cell wall isolate (5 x 10^6 particles/ml). MIP-2 release under these conditions was determined by ELISA. **B.** To further verify the specificity of anti-CDw17 on suppression of MIP-2 release by AEC’s in response to *P. carinii* β-glucan, epithelial cells were stimulated in parallel with either PC β-glucan (5 x 10^6 particles/ml) or LPS (1.0 µg/ml) in the presence or absence of anti-CDw17 (200 µg/ml), and MIP-2 release assessed by ELISA. Data points are expressed as mean ± SEM (* Denotes P<0.05, compared to control AECs incubated with glucan alone)

**Figure 6.** Incubation of AEC’s with either whole IgM or IgG/rIgG anti-CDw17 both significantly decrease MIP-2 production following PCBG stimulation. AEC’s were incubated with 200 µg/ml of either whole IgM anti-CDw17 or digested anti-CDw17 IgM (IgG and rIgG) during challenge with PC β-glucan and MIP-2 release determined by ELISA. In parallel, anti-CDw17 (IgM) was pre-incubated with solubilized lactosylceramide. Data points are expressed as mean ± SEM (* Denotes P<0.05, compared to control AEC’s incubated with glucan alone; ** Denotes P<0.05 comparing IgM anti-CDw17 and anti-CDw17 preincubated with lactosylceramide).

**Figure 7.** Effect of glycosphingolipid biosynthesis inhibitors on β-glucan induced MIP-2 generation from alveolar epithelial cells. **A.** AECs were incubated with either PDMP (20 µM) or NB-DNJ (200-400 µM) for 72 hours, challenged with PC β-glucan for a subsequent 18
hours, and MIP-2 release determined. **B.** To further verify specificity of the effect of inhibiting glycosphingolipid synthesis on suppression of MIP-2 release by AEC’s, epithelial cells were stimulated in parallel with either PC β-glucan (5 x 10^6 particles/ml) or LPS (1.0 µg/ml) in the presence or absence of NB-DNJ (400 µM). Data points are expressed as mean ± SEM. (* Denotes P<0.05 compared to AECs incubated with PC β-glucan alone)
REFERENCES


Figure 3

- MIP-2
- L32
- GAPDH

C  +PCBG
Figure 4

<table>
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Figure 5A

MIP-2 (pg/ml)

Glucan Treatment: - (Control), +

Antibody Treatment: NI-IgM, Anti-CDw17, Anti-CD11b, Anti-AsialoGM1

* denotes a significant difference compared to the control.
Figure 5B

The bar graph shows the MIP-2 (pg/ml) levels in different conditions:

- Glucan Alone
- Glucan + Anti-CDw17
- LPS Alone
- LPS + Anti-CDw17

The graph indicates a significant decrease in MIP-2 levels when Glucan is combined with Anti-CDw17, as indicated by the asterisk (*).
Figure 7A

The graph shows the concentration of MIP-2 (pg/ml) in response to different treatments. The x-axis represents the Glucan Treatment, with options including Control, PDMP (20 μM), NB-DNJ (200 μM), and NB-DNJ (400 μM). The y-axis represents the concentration of MIP-2 in pg/ml, ranging from 0 to 9000.

- The Control treatment without Glucan shows a low baseline level of MIP-2.
- Adding Glucan without treatment shows a significant increase in MIP-2 concentration.
- Treatment with PDMP (20 μM) reduces MIP-2 concentration compared to the Glucan control.
- Treatment with NB-DNJ (200 μM) and NB-DNJ (400 μM) further reduces MIP-2 concentration compared to PDMP.

Each bar represents the mean ± standard error of the mean (SEM). Asterisks indicate significant differences compared to the Glucan control.
Figure 7B

The graph shows the levels of MIP-2 (pg/mL) in different conditions:

- Glucan Alone
- Glucan + NB-DNJ
- LPS Alone
- LPS + NB-DNJ

The graph indicates a significant decrease in MIP-2 levels when Glucan is combined with NB-DNJ compared to Glucan Alone. This is denoted by an asterisk (*) on the graph.
Pneumocystis carinii cell wall β-glucan induces release of macrophage inflammatory protein-2 from alveolar epithelial cells via a lactosylceramide mediated mechanism
Peter Y. Hahn, Scott E. Evans, Theodore J. Kottom, Joseph E. Standing, Richard E. Pagano and Andrew H. Limper

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