Isolated *Pneumocystis carinii* Cell Wall Glucan Provokes Lower Respiratory Tract Inflammatory Responses

Robert Vassallo,* Joseph E. Standing,* and Andrew H. Limper.†

Macrophage-induced lung inflammation contributes substantially to respiratory failure during *Pneumocystis carinii* pneumonia. We isolated a *P. carinii* cell wall fraction rich in glucan carbohydrate, which potently induces TNF-α and macrophage-inflammatory protein-2 generation from alveolar macrophages. Instillation of this purified *P. carinii* carbohydrate cell wall fraction into healthy rodents is accompanied by substantial increases in whole lung TNF-α generation and is associated with neutrophil infiltration of the lungs. Digestion of the *P. carinii* cell wall isolate with zymolase, a preparation containing predominantly β-1,3 glucanase, substantially reduces the ability of this *P. carinii* cell wall fraction to activate alveolar macrophages, thus suggesting that β-glucan components of the *P. carinii* cell wall largely mediate TNF-α release. Furthermore, the soluble carbohydrate β-glucan receptor antagonists laminariheptaose and laminarin also substantially reduce the ability of the *P. carinii* cell wall to stimulate macrophage-inflammatory activation. In contrast, soluble α-mannose, a preparation that antagonizes macrophage mannose receptors, had minimal effect on TNF-α release induced by the *P. carinii* cell wall fraction. *P. carinii* β-glucan-induced TNF-α release from alveolar macrophages was also inhibited by both dexamethasone and pentoxifylline, two pharmacological agents with potential activity in controlling *P. carinii*-induced lung inflammation. These data demonstrate that *P. carinii* β-glucan cell wall components can directly stimulate alveolar macrophages to release proinflammatory cytokines mainly through interaction with cognate β-glucan receptors on the phagocyte.


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**Abbreviations used in this paper:** MIP, macrophage-inflammatory protein; gpA, glycoprotein A.

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Materials and Methods

General reagents were from Sigma (St. Louis, MO), unless otherwise specified. P. carinii f. sp. (forme specialis) carinii was originally obtained through the American Type Culture Collection (ATCC, Manassas, VA) and has been maintained in our immunosuppressed rat colony. L929 cells were also from ATCC. Ciprofloxacin was the generous gift of Dr. Barbara Painter of Miles Pharmaceuticals (West Haven, CT).

Generation of a P. carinii cell wall fraction

All animal studies were reviewed and approved by the Mayo institutional animal care and usage committee. P. carinii pneumonia was induced in Harlan Sprague-Dawley (HSD) rats by immunosuppression with dexamethasone, as we previously described (24, 25). Female rats (250 g; Harlan Sprague-Dawley (HSD) rats by immunosuppression with dexamethasone, or by the drug pentoxifylline, another agent that can inhibit mononuclear cell TNF-α release (21–23).

Recent studies from our group and by others indicate that whole P. carinii organisms can stimulate alveolar macrophages to release TNF-α most likely through interactions of P. carinii with distinct β-glucan receptors on macrophages (24, 25). Treatment of the organisms with immobilized polymyxin or macrophage stimulation in the presence of soluble polymyxin did not suppress the ability of whole P. carinii to induce macrophage TNF-α release, strongly eliminating endotoxin contamination as the source of this important inflammatory stimulus (24, 25). Although these studies clearly argue that P. carinii induces TNF-α generation from macrophages, it is not known whether the cell wall of P. carinii itself stimulates TNF-α release from macrophages, in the absence of intact viable organisms. Furthermore, the specific components of the cell wall of P. carinii that cause TNF-α release and their cognate receptors on alveolar macrophages have not yet been fully defined. If true, remnant P. carinii cell wall fragments may continue to incite lung inflammation even after the organisms are rendered nonviable by conventional antibiotics.

The current investigation was, therefore, undertaken to accomplish the following goals: 1) to isolate a cell wall fraction of P. carinii that is rich in β-glucan and to directly evaluate the ability of this component of the P. carinii cell wall to stimulate proinflammatory cytokine and chemokine release from alveolar macrophages; 2) to evaluate the role of macrophage mannose and β-glucan receptors in mediating this inflammatory response; 3) to determine whether treatment of alveolar macrophages with the glucocorticoid dexamethasone and pentoxifylline would alter any TNF-α response elicited by this P. carinii cell wall isolate.

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B/L). A total of 2 × 10^5 macrophages was plated per well in 96-well tissue culture plates, allowed to adhere for 60 min, and gently washed to remove any unattached cells. Subsequently, varying concentrations of the P. carinii cell wall isolate (1 × 10^6 to 1 × 10^9 particles/ml) were incubated with the alveolar macrophages for 12 h (37°C, 5% CO₂). Following incubation, the media was removed, centrifuged (10,000 × g for 5 min) to remove any particulate material or cells, and assayed for the presence of TNF-α, as described below.

To determine whether these P. carinii components also induce macrophage chemokine release, identical experiments were conducted to determine MIP-2 release from cultured alveolar macrophages in the presence of varying amounts of the P. carinii cell wall isolate. For comparison, parallel experiments were done using β-glucan from the related fungus Saccharomyces cerevisiae. Macrophages were obtained from pathogen-free rats and stimulated with varying concentrations of S. cerevisiae β-glucan (ranging from 100 to 600 μg/ml). Parallel experiments were conducted by incubating alveolar macrophages with varying concentrations of the glucan-rich P. carinii cell wall isolate (ranging from 0.1 to 1 × 10⁷ particles/ml). Following 12 h of incubation, the supernatants were harvested and the presence of MIP-2 determined by ELISA.

We further investigated whether this P. carinii cell wall isolate could be rendered inactive in stimulating cytokine release from alveolar macrophages by digestion with β-1,3 glucanase, thereby indicating that the observed response was secondary to the presence of β-glucan in the cell wall. To address this, the P. carinii cell wall preparations were digested with 0.5 mg/ml of zymolyase (zymolyase 20T; Seikagaku, Rockville, MD) for 1 h at 25°C before incubation with alveolar macrophages. Zymolyase is a preparation containing predominantly β-1,3 glucanase activity (24). As a control, the same amount of P. carinii cell wall isolate was sham treated in PBS without enzyme for an equal period of time and subsequently incubated with macrophages. Following 12 h of incubation, the media were harvested and assayed for the presence of TNF-α.

**Quantification of TNF-α and MIP-2**

The presence of TNF-α released from alveolar macrophages into the culture media was quantified using a sensitive and specific TNF-α ELISA, according to the manufacturer’s instructions (Genzyme, Cambridge, MA). In addition, TNF-α bioactivity was further analyzed by the L929 cytotoxicity assay, as previously reported (35). In brief, L929 cells were plated (2 × 10⁵) in 96-well plates and incubated overnight to achieve confluence using Earle’s MEM with 10% FCS, and 2 mM L-glutamine. The following day, the wells were replaced with fresh media containing actinomycin D (1 μg/ml) containing serial dilutions of either murine TNF-α standard (Genzyme) or unknown samples. After 20 h of incubation at 37°C and 5% CO₂, cytotoxicity was determined using a colorimetric XTT assay reagent (Boehringer Mannheim, Indianapolis, IN) at 490 nm. A standard concentration curve was prepared from the standards and used to determine the relative TNF-α concentrations of the samples, as in our previous studies (24, 37). MIP-2 levels in the supernatants were quantified using a sensitive and specific MIP-2 ELISA, according to the manufacturer’s instructions (BioSource International, Camarillo, CA).

**Role of macrophage β-glucan and mannose receptors in mediating TNF-α release in response to the isolated P. carinii cell wall fraction**

We next sought to determine the relative roles of macrophage β-glucan and mannose receptors in mediating TNF-α release in response to the isolated P. carinii cell wall fraction. Our prior studies have implicated β-glucan receptors and mannose receptors in mediating macrophage TNF-α generation in response to whole P. carinii organisms (24). Accordingly, studies were undertaken to competitively inhibit these same receptor systems during stimulation with the newly purified P. carinii cell wall isolate. To specifically determine whether macrophage mannose receptors mediate TNF-α release, macrophages were incubated with the soluble mannose receptor antagonist, α-mannan (derived from S. cerevisiae; 0.5 mg/ml; Sigma). We also incubated the addition of P. carinii cell wall isolate (5 × 10⁷ particles/ml) and throughout a subsequent 12-h incubation. In parallel, the role of macrophage β-glucan receptors was evaluated by incubating macrophages with laminarin (Sigma) or laminariheptaose (Seikagaku America, Fajamsville, MD), which competitively inhibits macrophage β-glucan receptors (24, 38, 39). Specifically, alveolar macrophages were incubated with the addition of laminarin or laminariheptaose for 45 min before addition of the P. carinii cell wall isolate (5 × 10⁷ particles/ml), and throughout a subsequent 12 h of coincubation. Subsequently, the media were removed and assayed for TNF-α using the L929 assay.

**Determination of alveolar macrophage viability following incubation with the P. carinii cell wall isolate and with receptor antagonists**

Additional experiments were undertaken to determine whether the P. carinii cell wall isolate or any of the competitive receptor antagonists tested impaired macrophage viability. To address this, alveolar macrophages were plated (200,000/well) on a 96-well plate, allowed to adhere for 60 min, and gently washed. The P. carinii cell wall isolate was next added (10⁶–10⁷ particles/ml) and incubated for 16 h with the macrophages. Similarly, macrophages were also incubated with either laminarin, laminariheptaose, or α-mannan at concentrations of 1–2 mg/ml. Following incubation, macrophage viability was assessed using theXTT reagent system according to the manufacturer’s instructions (Boehringer Mannheim, Indianapolis, IN).

**Effect of dexamethasone and pentoxifylline on macrophage TNF-α release induced by the P. carinii cell wall isolate**

Subsequently, we evaluated the effects of the potent glucocorticoid dexamethasone (Fujisawa USA, Deerfield, IL), and the phosphodiesterase inhibitor pentoxifylline (Sigma), on P. carinii cell wall-induced TNF-α release from alveolar macrophages. Macrophages (2 × 10⁵) from pathogen-free HSD rats were preincubated with varying concentrations of dexamethasone (0.1–500 μM) or pentoxifylline (3–30 μg/ml) for 1 h before the addition of 5–7.5 × 10⁷ P. carinii cell wall particles and throughout a subsequent 12-h incubation. Afterward, the supernatant was removed and assayed for TNF-α by ELISA and L929 assay.

**Statistical analysis**

All reported experiments were repeated on multiple occasions using separate batches of animals, alveolar macrophages, and P. carinii cell wall isolate. All data are expressed as mean ± SEM. Differences between experimental and control data groups were determined using two-tailed Student’s t tests for normally distributed variables. For nonparametric variables, data were analyzed using the Mann-Whitney U test. Statistical testing was performed on the Statview II statistical package (Abacus Concepts, Berkeley, CA) using a Power Macintosh personal computer. Statistical differences between data were considered to be significant if p was <0.05.

**Results**

**Generation and initial characterization of a P. carinii cell wall isolate enriched in glucans**

Using the cell wall isolation procedure described, ~1 mg (equivalent to 10⁶ cell wall particles/ml, following ultrasonication) of P. carinii cell wall isolates was obtained from pooled products ranging from 20 to 40 rats. The final cell wall fraction 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, although not completely, hydrolyzed with 2 M HCl, thereby releasing 82% of its content as α-glucose measured by the glucose oxidase method. Thus, the majority of this material represents P. carinii-derived glucose polymer, namely glucans. The remainder is most likely composed of incompletely hydrolyzed polysaccharides containing glucose and other sugars, as well as small amounts of residual protein. This P. carinii cell wall isolate contained <0.125 U/ml of soluble endotoxin when assessed by the Limulus amebocyte lysate assay. Taken together, these findings strongly indicate that we have isolated a P. carinii carbohydrate cell wall fraction substantially enriched in glucans.

**The P. carinii cell wall isolate induces lung inflammation in rodents**

We next sought to evaluate whether the P. carinii cell wall isolate would induce lung inflammation and TNF-α generation in a living host, in the absence of intact microorganisms. Intratracheal challenge with the P. carinii cell wall isolate stimulated substantial TNF-α release in the animal lungs (Fig. 1). Mice challenged with 3.2 × 10⁶ P. carinii cell wall particles/ml produced...
Each data point represents the mean ± SD of two experiments performed in duplicate.

These observations indicate that the P. carinii cell wall can directly stimulate TNF-α release from alveolar macrophages. Alveolar macrophages (2 × 10^5/well) were incubated with varying concentrations (1 × 10^6 to 1 × 10^8 particles/ml) of the glucan-rich P. carinii cell wall isolate for 12 h. Subsequently, TNF-α release into the medium was measured by the L929 bioactivity assay. Maximal TNF-α release occurred with 2.5 × 10^7 particles/ml (*, p < 0.05 compared with unstimulated macrophages). Interestingly, this TNF-α response declines when alveolar macrophages are incubated with higher concentrations of P. carinii cell wall isolate. Each data point represents the mean ± SD of two experiments performed in duplicate.

145.2 ± 118-fold more TNF-α compared with mice treated with saline buffer only (p = 0.032). It should again be emphasized that this response was not the result of endotoxin contamination, since Limulus amebocyte lysate assays on the P. carinii cell wall isolate failed to detect any measurable soluble endotoxin.

Furthermore, the TNF-α generation observed in mice receiving P. carinii cell wall isolate also had substantial accompanying neutrophil infiltration into the lung. Total lung lavage contained 38.1 ± 4.2% neutrophils from mice challenged with 3.2 × 10^6 per ml P. carinii cell wall particles compared with 0.2 ± 0.3% neutrophils in control mice receiving saline alone (p = 0.0026).

The P. carinii cell wall isolate is a potent stimulant of TNF-α and MIP-2 release from cultured alveolar macrophages

To further determine the mechanisms by which the P. carinii cell wall isolate mediates inflammatory signaling in the lung, we assessed whether this P. carinii component would induce TNF-α and MIP-2 release from cultured alveolar macrophages. Accordingly, macrophages were obtained from pathogen-free rats and stimulated with varying concentrations of the P. carinii cell wall isolate (Fig. 2). Although unstimulated macrophages released only 14 ± 5 pg/ml of TNF-α into the medium, alveolar macrophages incubated with 2.5 × 10^7 particles/ml of the P. carinii cell wall isolate generated a maximum of 4180 ± 735 pg/ml (p = 0.002 compared with unstimulated control macrophages). To further exclude the possibility of endotoxin contamination, additional experiments were also performed in the presence of the endotoxin-neutralizing agent polymyxin B (2.5 µg/ml), with measured TNF-α induced by the P. carinii cell isolate, which did not differ from the data presented in Fig. 1 (data not shown). Interestingly, this maximal TNF-α response declined when alveolar macrophages were incubated with higher concentrations of P. carinii cell wall isolate. Indeed, incubations with large concentrations of P. carinii cell wall isolate (1 × 10^8 particles/ml) induced only 69.3 ± 18.6 pg/ml of TNF-α (not significantly different compared with control). Macrophage viability was consistently greater than 95% in the presence of these concentrations of the glucan-rich cell wall isolate.

Digestion of P. carinii cell wall isolates with zymolase substantially inhibits macrophage TNF-α release

We next investigated whether the P. carinii cell wall isolate could be rendered inactive by digestion with a β-1,3 glucanase preparation, thus supporting our hypothesis that the observed macrophage cytokine responses were largely a result of stimulation by β-glucan in the P. carinii cell wall. To test this, several concentrations of P. carinii cell wall isolate ranging from 0.1 to 1 × 10^7 particles/ml...
were treated with zymolyase (0.5 mg/ml, for 1 h at 25°C), a preparation that contains largely β-glucanase activity (24). Subsequently, equal particle numbers of digested and undigested P. carinii cell wall isolates were incubated with alveolar macrophages overnight, and the release of TNF-α in the media was quantified using the L929 assay. Digestion of the P. carinii cell wall isolate resulted in a substantial loss of the macrophage-stimulatory activity of this P. carinii component (Fig. 4). While 1 × 10^7 particles/ml of undigested P. carinii cell wall isolate induced 1972 ± 498 pg/ml of TNF-α, following digestion, the same number of particles induced only 420 ± 8 pg/ml of TNF-α (p = 0.048). This observation strongly suggests that β-glucan components are largely responsible for macrophage TNF-α release following stimulation with the P. carinii cell wall isolate. Furthermore, the ability of zymolyase to abolish most of the observed TNF-α release additionally supports the contention that this effect is not secondary to contaminating endotoxin, because endotoxin is not inactivated by this enzyme preparation.

Soluble β-glucan receptor antagonists, but not mannose receptor antagonists, suppress alveolar macrophage TNF-α release in response to the P. carinii cell wall isolate

Our previous studies have shown that TNF-α release from alveolar macrophages incubated with whole P. carinii organisms can be suppressed by yeast β-glucan, through the competitive inhibition of macrophage β-glucan receptors (24). Those studies further indicated that yeast α-mannan, an antagonist of macrophage mannose receptors, had no significant effect on P. carinii-induced macrophage TNF-α release. Consistent with these earlier observations, incubation of macrophages in the presence of either laminarin (0.5 mg/ml) or laminariheptaose (0.5 mg/ml), which are soluble carbohydrate antagonists of macrophage β-glucan receptors, significantly reduced macrophage TNF-α generation following challenge with the glucan-rich P. carinii cell wall isolate (Fig. 5). In the presence of laminarin, TNF-α release was inhibited by 61.6 ±
4.8% \( (p = 0.0001 \) compared with the alveolar macrophages stimulated with \( P. \) carinii cell wall isolate alone). Similarly, TNF-\( \alpha \) release was also suppressed with the heptaglucosaccharide laminariheptaose by 37.8 \( \pm \) 9.1% \( (p = 0.035 \) compared with control). In contrast, incubation of macrophages with yeast \( \alpha \)-mannan, which antagonizes macrophage mannose receptors, did not cause any significant suppression of macrophage TNF-\( \alpha \) release induced by the \( P. \) carinii cell wall isolate \( (p = 0.36 \) compared with macrophages incubated without \( \alpha \)-mannan; Fig. 5). Therefore, these data strongly indicate that macrophage release of TNF-\( \alpha \) following interaction with \( P. \) carinii cell wall predominantly occurs through interactions with macrophage \( \beta \)-glucan receptors.

It is highly unlikely that laminarin or laminariheptaose resulted in a nonspecific down-regulation of macrophage ability to secrete TNF-\( \alpha \). Viability studies using the XTT reagent (Boehringer Mannheim) performed on macrophages incubated with concentrations of laminarin and laminariheptaose ranging from 0.5 to 2 mg/ml demonstrated \( >95\% \) viability of the macrophages following 24 h of incubation. Furthermore, the absence of inhibition of macrophage TNF-\( \alpha \) induction by soluble mannose receptor antagonists, at concentrations known to interfere with gpA-macrophage interactions \( (24, 40) \), further supports our contention that residual mannose-rich proteins such as gpA in the cell wall preparation are not likely responsible for the observed macrophage-inflammatory signaling induced by this \( P. \) carinii isolate.

Dexamethasone and pentoxifylline reduce TNF-\( \alpha \) release from alveolar macrophages stimulated with the glucan-rich \( P. \) carinii cell wall

Attenuation of the TNF-\( \alpha \) release induced by \( P. \) carinii is desirable for controlling lung inflammation during severe \( P. \) carinii pneumonia. We, therefore, evaluated the potential roles of dexamethasone and pentoxifylline in modifying macrophage TNF-\( \alpha \) responses to the \( P. \) carinii cell wall isolate in vitro. Concentrations of dexamethasone equal to, or greater than, 1 \( \mu \)M resulted in suppression of the maximal observed TNF-\( \alpha \) release by 43.8 \( \pm \) 8.4% \( (p = 0.0001 \) compared with the TNF-\( \alpha \) release in the absence of dexamethasone). No significant difference was observed whether TNF-\( \alpha \) was assayed using either the L929 bioassay or ELISA. Maximum inhibition was observed with a dexamethasone concentration of 125 \( \mu \)M. Alveolar macrophages incubated with 125 \( \mu \)M dexamethasone before the addition of the \( P. \) carinii cell wall isolate released 66.7 \( \pm \) 5.9% less TNF-\( \alpha \) (Fig. 6; L929 assay) compared with macrophages challenged with the cell wall isolate in the absence of dexamethasone \( (p = 0.001) \).

Similarly, the phosphodiesterase inhibitor pentoxifylline \( (\geq 15 \) \( \mu \)g/ml) also resulted in significant suppression of macrophage TNF-\( \alpha \) release induced by the \( P. \) carinii cell wall isolate. TNF-\( \alpha \) release by the \( P. \) carinii cell wall was reduced by 34 \( \pm \) 1.7% when alveolar macrophages were incubated with 15 \( \mu \)g/ml of pentoxifylline \( (p = 0.022 \) compared with macrophages incubated without pentoxifylline). Maximal TNF-\( \alpha \) suppression was observed with 30 \( \mu \)g/ml of pentoxifylline, which decreased the maximal observed TNF-\( \alpha \) release by 44 \( \pm \) 13% \( (p = 0.0013 \) compared with macrophages incubated in the absence of pentoxifylline). A similar response was observed whether TNF-\( \alpha \) was measured with L929 or by ELISA \( (p = 0.02) \).

Discussion

\( P. \) carinii pneumonia remains a common clinical problem in immunocompromised hosts, both in patients with AIDS and with other conditions. Understanding host defense mechanisms that participate in resolution of this infection is critical to not only further our understanding of the pathogenesis of \( P. \) carinii pneumonia, but also to develop new therapeutic approaches in the management of this important infection. In this study, we characterized the ability of a newly generated glucan-rich \( P. \) carinii cell wall isolate to stimulate TNF-\( \alpha \) release from alveolar macrophages. These investigations demonstrate for the first time that the cell wall of \( P. \) carinii, in the absence of intact viable microorganisms, can directly promote lung inflammation. Furthermore, the TNF-\( \alpha \)-inflammatory response induced by the \( P. \) carinii cell wall is mediated principally by \( \beta \)-glucan components interacting with cognate receptors on the surface of alveolar macrophages. We additionally
demonstrate that both dexamethasone and pentoxifylline significantly suppress macrophage TNF-α release induced by this *P. carinii* β-glucan isolate.

Accumulating evidence indicates that TNF-α provides important functions during *P. carinii* pneumonia. In the lower respiratory tract, TNF-α is principally produced by alveolar macrophages (41). The central importance of TNF-α in host defense during *P. carinii* pneumonia has been convincingly demonstrated in animal models of infection lacking functional TNF-α or TNF-α receptors (13–15). When challenged with *P. carinii*, these animals consistently develop chronic infection characterized by significantly delayed clearance of *P. carinii* organisms. The precise manner in which TNF-α facilitates resolution of *P. carinii* infection from the host has not been completely elucidated, but recent studies indicate that TNF-α promotes the expression of cell surface adhesion molecules, such as ICAM-1, which strongly facilitates recruitment of inflammatory cells into the lung (16). Furthermore, TNF-α is also critical in the activation of NK cells and CD8+ T lymphocytes, and for regulation of class I MHC expression (42). Still other investigations suggest that TNF-α may also be directly toxic to *P. carinii* by suppressing carbohydrate metabolism in the organism (43, 44).

Although TNF-α substantially aids in the elimination of *P. carinii*, excessive quantities of TNF-α are also deleterious to respiratory function of the host. TNF-α induces endothelial cell permeability, promotes edema formation, and enhances exuberant inflammatory cell recruitment in the lungs, each of which has been associated with further impairment of gas exchange (16, 45, 46).

Earlier work demonstrates that neutrophil infiltration in the lungs of patients with *P. carinii* pneumonia is a predictor of respiratory failure and death (1, 3). This observation is supported clinically by the effectiveness of glucocorticoids as adjunctive treatment for patients with *P. carinii* pneumonia complicated with respiratory failure (19, 20, 47). Although glucocorticoids have been proven to decrease morbidity and mortality in severe *P. carinii* pneumonia, the mechanisms by which this occurs are not fully known. It is likely that the benefit achieved from glucocorticoids relates, at least in part, to controlling excessive inflammation induced by *P. carinii* cell wall components. In this light, we currently demonstrate that dexamethasone, a potent glucocorticoid, effectively suppresses alveolar macrophage TNF-α release induced by *P. carinii* cell wall β-glycans. We further demonstrate that pentoxifylline, a drug that has been shown to increase survival in models of murine endotoxic shock and other animal models of acute lung injury mediated by TNF-α (48, 49), also inhibits TNF-α release induced by *P. carinii* cell wall components. These observations further suggest that pentoxifylline may represent another therapeutic adjunct in the management of severe *P. carinii* pneumonia.

Although prior studies clearly document TNF-α release from alveolar macrophages challenged with whole *P. carinii* organisms, concerns have continued to arise that macrophage stimulation may be the result of contaminating host proteins or other molecules adherent to *P. carinii* (24, 50, 51). In the current study, greater than 95% of *P. carinii* and host proteins were removed from the cell wall carbohydrate fraction, yielding a preparation that was far more potent in inducing macrophage-inflammatory responses. Furthermore, we strongly believe that the stringent washes and neutralization conditions undertaken in the preparation of this cell wall fraction fully eliminated endotoxin contamination, thereby excluding this important confounding variable. Moreover, generation of this glucan-rich *P. carinii* cell wall isolate provides a simplified, accurate, and reliable system for investigation of direct *P. carinii* cell wall effects on macrophage-inflammatory signaling.

Consistent with our prior investigations using whole organisms, the current study indicates that *P. carinii* triggers macrophage-inflammatory activation through interaction with β-glucan receptors, thus representing an important receptor system mediating recognition of this pathogen (24). A number of other studies additionally demonstrate that macrophage mannose receptors interacting with *P. carinii* gpA provide an active pathway for *P. carinii* uptake by alveolar macrophages (40, 52). Nevertheless, the soluble mannose receptor antagonist α-mannan was not associated with any appreciable alteration in macrophage TNF-α release, either in our prior studies using whole *P. carinii* organisms (24), or in the current study using the cell wall isolate. In a similar manner, macrophage oxidative responses to *P. carinii* have also been reported to be mediated through β-glucan components on the organism’s surface (53). These observations indicate that while macrophage mannose receptors may represent an important mechanism of macrophage uptake of *P. carinii*, they may play a substantially lesser role in stimulating macrophage-inflammatory responses toward this organism.

Similar to those purified from related fungi, the glucan-rich cell wall isolate from *P. carinii* was derived in particulate form. Investigations with other purified fungal β-glucans have demonstrated that particulate and/or poorly soluble β-1,3 glucans directly induce mononuclear cells to release TNF-α and IL-1β in vitro (10, 54). It has been postulated that particulate (but not soluble) β-glucans elicit these responses because of cross-linking or immobilization of β-glucan receptors (55).

Similar to what was observed with β-glucan from the nonpathogenic fungus *S. cerevisiae*, maximal TNF-α release occurred with moderate concentrations of β-glucan (100–200 μg/ml), whereas higher concentrations of β-glucan (>500 μg/ml) caused apparent suppression of TNF-α release (12). Our prior studies performed with *S. cerevisiae* β-glucan demonstrate that suppression of TNF-α activity by high concentrations of β-glucan was partially mediated by the particulate β-glucan-binding soluble TNF-α, through the lectin-binding domain of the cytokine, rendering the TNF-α less available for measurement (12). It is also possible that high concentrations of β-glucan may suppress TNF-α release by other mechanisms, including regulation of cytokine gene expression (unpublished observations). The biological significance of this bimodal response is not clear. It is possible that, in the presence of large amounts of *P. carinii* organisms in the airways, significant quantities of TNF-α are prevented from exerting an effect on the host by binding to the fungal cell walls. Potentially, the inhibition of TNF-α activity by large amounts of β-glucan may represent a pathway by which *P. carinii* gains survival advantage in the airways of infected individuals. It is interesting that no apparent suppression of MIP-2 release was observed with comparable doses of β-glucan from either *P. carinii* or *S. cerevisiae*. Thus, the immunomodulatory effects of β-glucan may be directed specifically to TNF-α release, rather than a general suppression of immune activation of alveolar macrophages.

Our study represents the first attempt at isolating and characterizing the activity of a β-glucan-enriched cell wall fraction from *P. carinii*. Digestion of this *P. carinii* product with β-1,3 glucanase results in substantial, albeit not complete, inhibition of its stimulatory activity. It might be postulated that the inability to completely suppress TNF-α-stimulatory activity by zymolyase digestion could be the result of trace amounts of *P. carinii* proteins contaminating the preparation. In particular, the mannose-rich *P. carinii* gpA surface glycoprotein would seem an unlikely culprit for mediating this residual activity for the reasons discussed above. Alternatively, there may be other stimulatory components in the *P. carinii* cell wall that resist digestion with β-glucanase, such as α-glucans and chitins, although these have yet to be fully characterized within the *P. carinii* cell wall.
In summary, our study demonstrates that the cell wall of *P. carinii* is a potent stimulant of TNF-α release by alveolar macrophages. This response is largely mediated by the rich β-glucan component of the cell wall and is inhibited by digestion of the *P. carinii* cell wall isolate with β-glucanase. This TNF-α release by alveolar macrophages is mainly mediated via interaction of the *P. carinii* cell wall components with macrophage β-glucan receptors. In addition, our study demonstrates that the cell wall of *P. carinii* is a potent inducer of MIP-2 release from alveolar macrophages and causes significant lower respiratory tract inflammation and neutrophil recruitment in rodents. Finally, we demonstrate that TNF-α release by alveolar macrophages in response to the *P. carinii* cell wall components may be partially suppressed by both dexamethasone and pentoxifylline.

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