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Isolated *Pneumocystis carinii* Cell Wall Glucan Provokes Lower Respiratory Tract Inflammatory Responses[1]

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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 neutrophilic infiltration of the lungs. Digestion of the *P. carinii* cell wall isolate with zymolyase, 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 isolate to stimulate macrophage-inflammatory activation. In contrast, soluble α-mannan, 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. *The Journal of Immunology,* 2000, 164: 3755–3763.

*P*neumocystis carinii is an opportunistic pathogen that continues to induce severe and often lethal pneumonia in immunosuppressed patients with AIDS, hematological and solid malignancies, organ transplantation, and as a consequence of sustained corticosteroid and cytotoxic therapy for inflammatory disorders (1, 2). Morbidity and mortality during *P. carinii* pneumonia are largely related to exuberant neutrophilic lung inflammation and accompanying diffuse alveolar injury (2–5). Although recently classified as a fungus, the structure of the *P. carinii* cell wall and interaction of *P. carinii* surface molecules with host alveolar inflammatory cells have not yet been fully characterized (6).

Accumulating evidence suggests that *P. carinii* assembles a cell wall rich in fungal β-glucans (7). β-glucans are major structural components of many related fungal cell walls, chemically consisting of 1,3-linked β-D-glucopyranosyl residues with differing amounts of 1,6-linked β-D-glucopyranosyl side chains of varying length and distribution frequency (8, 9). Purified β-glucans from several fungal species phylogenetically related to *P. carinii* have been shown to stimulate the release of proinflammatory cytokines, most notably TNF-α and IL-1β from cultured mononuclear cells (10–12). Little is known about the ability of β-glucan to induce chemokine release from inflammatory cells. Such responses are thought to represent essential components of host immune responses to invading fungal pathogens.

The release of TNF-α by alveolar macrophages in response to *P. carinii* appears to be critical for elimination of organisms from the host. Animals challenged with *P. carinii* exhibit substantially impaired clearance of organisms when TNF-α activity is inhibited by neutralizing Abs or when TNF-α receptors are genetically deleted (13, 14). Even immunocompetent mice fail to effectively clear *P. carinii* after treatment with recombinantly expressed TNF-α inhibitor (15). Although TNF-α has an essential role in the elimination of *P. carinii*, it is also likely that excessive production of TNF-α may have deleterious effects on host respiratory function. TNF-α enhances inflammatory cell recruitment, particularly neutrophils, through induction of adhesion molecules (16), and promotes edema formation by increasing endothelial cell permeability (17). Exuberant inflammatory responses from excessive and sustained TNF-α release may promote respiratory failure and death in certain patients with *P. carinii* pneumonia (1, 3, 5). In addition to TNF-α, alveolar macrophages secrete a variety of chemokines that enhance neutrophil recruitment to the lungs (17). Of particular interest is the potent chemokine macrophage-inflammatory protein-2 (MIP-2).[3] MIP-2 is the rodent equivalent of human IL-8, and is a potent chemoattractant to neutrophils (18).

In this setting, potent anti-inflammatory medications including glucocorticoid agents have been shown to improve outcomes and reduce mortality as adjunctive therapy in severe *P. carinii* pneumonia complicated by respiratory failure (19, 20). Even though steroids are an established component of the pharmacological management of severe *P. carinii* pneumonia, the mechanisms by which these drugs reduce mortality and improve outcomes in *P. carinii* pneumonia have not been fully elucidated. We postulate that stimulation of macrophage TNF-α release by active components of the organism's cell wall should be inhibited by the potent

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

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glucocorticoid 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.

Materials and Methods

Materials

General reagents were from Sigma (St. Louis, MO), unless otherwise specified. *P. carinii f. sp. (formae 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, 26, 27). Female rats (250 g; HSD, Indianapolis, IN) received drinking water containing dexamethasone (2 mg/L), tetracycline (500 mg/L), and nystatin (200,000 U/L) ad libitum. Each week, the animals additionally received oral ciprofloxacin (0.45 g/L) for 2 consecutive days to further reduce bacterial infections (27). After 1 wk, rats were anesthetized and inoculated with 500,000 *P. carinii* organisms by intratracheal injection. Six weeks later, the rats were sacrificed by lethal i.p. injection of phenobarbital and the lungs were removed. Both lungs were homogenized in a Stomacher Tissue Blender (Tekmar, Madison, WI) for 10 min, and *P. carinii* organisms were obtained from this homogenate by filtration through 10-μm filters (28). To exclude the presence of other infectious agents, isolates were routinely stained (Diff Quick Modified Wright-Giemsa stain; Dade Diagnostics, Aguada, PR), and selected isolates were cultured to exclude concurrent infection with bacteria or fungi. Isolates with significant contamination of other microorganisms were discarded.

Next, we performed a cell wall isolation procedure adapted from those previously reported to generate a β-glucan-enriched cell wall fraction in other fungal species (29–31). First, *P. carinii* organisms were autoclaved for 120°C, 20 min, to denature proteins. Ultrasonication of the organisms was then performed (200 W for 3 min, six times) and washed three times with PBS. Next, lipids and proteins were extracted by soaking in chloroform/methanol (2:1) for 2 h. Additional protein and short glycoconjugates were removed by alkaline extraction using 1 N sodium hydroxide over 1 h.

The insoluble fraction that remained was washed several times with deionized water until neutral and subsequently treated with ethanol, acetone, and diethyl ether extractions to dehydrate and fully remove lipids from the products.

Extensive measures were undertaken to eliminate contamination of the cell wall preparations with endotoxin. The *P. carinii* cell wall isolate was washed four times with sterile, 150 mM sodium chloride solution containing 10 μg/ml polymyxin B (Sigma). Additionally, the cell wall preparations were washed with a 1% SDS (Sigma) solution to solubilize residual protein and lipids and subsequently washed another three times with 150 mM sodium chloride to remove residual SDS. The *P. carinii* cell wall fractions were recovered from each wash by centrifugation (1400 × g × 15 min), and the final wash assayed for endotoxin using a modified *Limulus* amebocyte lysate assay (BioWhittaker, Walkersville, MD) with a lower limit of sensitivity of 0.125 U/ml. As an additional biological assessment of endotoxin contamination, selected supernatants from these washes were also incubated with alveolar macrophages for 12 h and assayed for TNF-α release. Following polymyxin B washes, the levels of endotoxin were consistently less than 0.125 U/ml. To further minimize the potential effect of contaminating endotoxin in the cell wall preparation, incubations of the *P. carinii* cell wall with alveolar macrophages were also done in media containing 2.5 μg/ml polymyxin B to neutralize any residual endotoxin.

Initial characterization of the preparation was undertaken as follows. First, the carbohydrate content of the isolated *P. carinii* cell wall fraction was determined using the orcinol-sulfuric acid sugar method (32). In brief, a sample of *P. carinii* cell wall fraction was hydrolyzed by boiling in 1 ml of 2 M HCl at 100°C over 3 h. Total carbohydrate content of the solubilized fraction was determined by reaction with orcinol dissolved in ice-cold concentrated sulfuric acid. The colorimetric reaction was developed at 80°C over 15 min. Standard concentrations of D-glucose were assayed as concurrent references, with the absorbances determined at 405 nm. Protein content was also evaluated on the solubilized fraction in parallel using the BCA reagent with standard albumin concentrations as references (Pierce, Rockford, IL). To quantitate glucose content, a sample of *P. carinii* cell wall fraction was hydrolyzed with 2 M HCl (boiling at 100°C), and glucose content on the solubilized fraction was measured by the glucose oxidase method using a commercial assay system (Sigma).

Induction of lung TNF-α release and inflammation following intratracheal challenge with P. carinii cell wall isolates

To next characterize the ability of the *P. carinii* cell wall isolate to induce lung inflammation in whole animals, experiments were undertaken to measure TNF-α release and lung inflammatory cell traffic following intratracheal instillation of healthy mice with *P. carinii* cell wall preparations. The *P. carinii* cell wall isolate was prepared as described and meticulously handled to prevent any contamination with endotoxin. Normal female ICR mice (Harlan, Indianapolis, IN) were used for this study. Although the *P. carinii* cell wall isolate was derived from *P. carinii f. sp. carinii* propagated in rats, mice were selected because of the very limited amounts of purified *P. carinii* cell wall isolate available for these whole animal challenges. Accordingly, under sterile conditions, mice were anesthetized with phenobarbital (i.p. injection of 50 mg/kg body weight), and the trachea exposed by dissection of the skin and strap muscles of the neck. Before intratracheal injection, the *P. carinii* cell wall isolate was suspended in sterile physiological saline by sonication, and quantified by hemocytometer counting. Varying concentrations of *P. carinii* cell wall isolate ranging from 3.2 × 10³ particles to 3.2 × 10⁶ were intratracheally injected into each mouse. Equal numbers of mice were used as controls and received physiological saline alone. Following 16 h, the mice were sacrificed and lavaged with 10 ml of cold saline. Cells were recovered from the lavage fluid by centrifugation, and cytopreparation smears were prepared. Cellular differentials were determined on 200 contiguous cells (33). The supernatants were concentrated using Centriprep concentrators (Amicon, Beverly, MA) and TNF-α in the lavage supernatants quantified using the L929 bioactivity assay described below.

TNF-α and MIP-2 release from alveolar macrophages stimulated with the P. carinii cell wall isolate

To further determine whether the isolated cell wall components of *P. carinii* would directly activate macrophage-inflammatory responses, normal alveolar macrophages were incubated with varying concentrations of the *P. carinii* cell wall isolate. To accomplish this, macrophages were collected by whole lung lavage of pathogen-free HSD rats. Smears consistently showed more than 95% alveolar macrophages in the lavage (34). Following centrifugation of the lavage (400 × g for 10 min), recovered cells were suspended in mixed media (RPMI, medium 199, 2 mM L-glutamine, 10,000 U penicillin/L, 1 mg streptomycin/L, and 25 μg/ml amphotericin

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 1×10^8 particles/ml) were incubated with the alveolar macrophages for 12 h (37°C, 5% CO_2). Following incubation, the media was removed, centrifuged ($10,000 \times g$ 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 1×10^7 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 (4×10^4/well) in 96-well plates and permitted to adhere 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% CO_2, cytotoxicity was determined using a colorimetric XTT assay reagent (Boehringer Mannheim, Indianapolis, IN) (36). Absorbances were determined 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 rather than 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), for 45 min before the addition of *P. carinii* cell wall isolate (5×10^6 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, Ijamsville, MD), which competitively inhibits macrophage β-glucan receptors (24, 38, 39). Specifically, alveolar macrophages were incubated with 0.5 mg/ml of laminarin or laminariheptaose for 45 min before addition of the *P. carinii* cell wall isolate (5×10^6 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 (10^6–10^8 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 the XTT reagent system according to the manufacturer's instructions (Boerhinger 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 (2×10^5) 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 $\times 10^6$ *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 $\pm$ 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 Macintoch 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 10^8 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 D-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 $\times 10^6$ *P. carinii* cell wall particles/ml produced
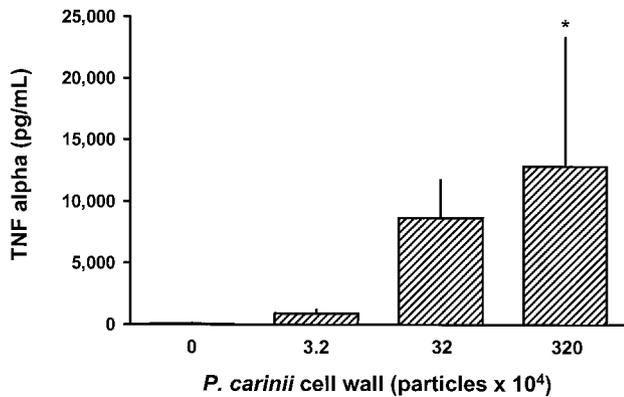
FIGURE 1. *P. carinii* cell wall isolate induces lung inflammation in rodents. Varying concentrations of the glucan-rich *P. carinii* cell isolate (3.2×10^3 to 3.2×10^6) were intratracheally injected into normal mice. TNF-α release into the lung lavage was measured by the L929 bioactivity assay. Mice challenged with 3.2×10^6 *P. carinii* cell wall particles/ml produced significantly greater TNF-α compared with mice treated with saline buffer only (∗, $p < 0.05$ compared with unstimulated macrophages). Each data point represents the mean $\pm$ SD of two experiments performed in duplicate.

FIGURE 2. *P. carinii* cell wall induces 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 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 $\pm$ 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 $58.1 \pm 4.2\%$ neutrophils from mice challenged with 3.2×10^6 per ml *P. carinii* cell wall particles compared with $0.2 \pm 0.3\%$ neutrophils in control mice receiving saline alone ($p = 0.0026$). Therefore, the *P. carinii* cell wall glucan-rich isolate induces both substantial lung cytokine and lung neutrophil influx in the absence of intact microorganisms.

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 ($2.5 \mu g$/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.
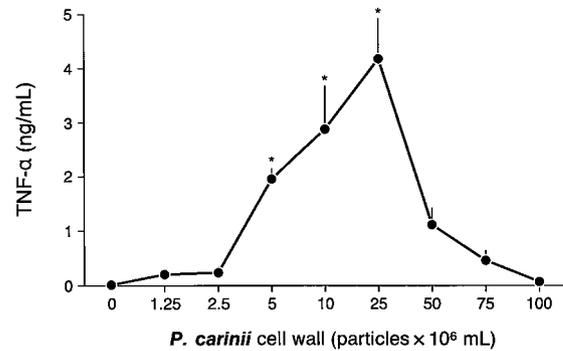
These observations indicate that the *P. carinii* cell wall can directly stimulate TNF-α release from alveolar macrophages at low and intermediate concentrations, while large concentrations are associated with suppressed TNF-α release. It is noteworthy that such findings are entirely consistent with our prior studies of macrophage TNF-α release in response to fungal β-glucan purified from *S. cerevisiae* (12). Thus, the glucan-rich *P. carinii* cell wall induces TNF-α generation from rat alveolar macrophages in a dose-dependent manner parallel to that of β-glucan derived from other phylogenetically related fungal species.

Similarly, release of MIP-2 from alveolar macrophages cultured in the presence of varying amounts of the *P. carinii* cell wall isolates and varying concentrations of *S. cerevisiae* β-glucan was examined. Macrophages stimulated with *S. cerevisiae* β-glucan at a concentration $>50 \mu g$/ml produced substantially more MIP-2 than unstimulated macrophages (Fig. 3a). Maximally, a concentration of $100 \mu g$/ml stimulated 157.9 ± 11.32 ng/ml of MIP-2 ($p = 0.0001$ compared with the unstimulated macrophages). Larger doses of *S. cerevisiae* β-glucan (up to $600 \mu g$/ml) did not result in further augmentation of MIP-2 release. In contrast to prior studies that demonstrated suppression of TNF-α release in response to high concentrations ($>500 \mu g$/ml) of *S. cerevisiae* β-glucan, there was no decrease in MIP-2 release with greater amounts of *S. cerevisiae* β-glucan. Stimulation of alveolar macrophages with the *P. carinii* cell wall resulted in a very similar response (Fig. 3b). Whereas unstimulated macrophages released only 2.3 ng/ml MIP-2, macrophages stimulated with $>1 \times 10^7$ particles/ml released significantly greater amounts of MIP-2 ($p = 0.0001$ for all concentrations of *P. carinii* cell wall tested compared with the negative control; Fig. 3b).

Digestion of P. carinii *cell wall isolates with zymolyase 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
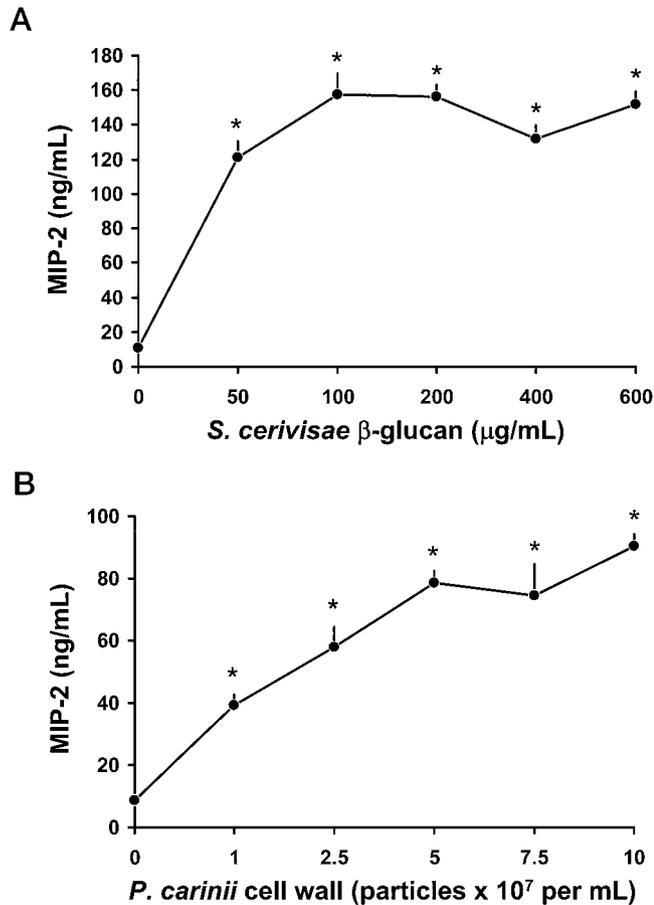
FIGURE 3. β-Glucan from *S. cerevisiae* and the *P. carinii* cell wall induces MIP-2 release from alveolar macrophages. *A*, Alveolar macrophages (2×10^5/well) were incubated with varying concentrations (50–600 μg/ml) of *S. cerevisiae* β-glucan for 12 h. Subsequently, MIP-2 release into the medium was measured by ELISA. Maximal MIP-2 release occurred with 100 μg/ml *S. cerevisiae* β-glucan (∗, $p < 0.001$ compared with unstimulated macrophages). Each data point represents the mean ± SD of two experiments performed in triplicate. *B*, Alveolar macrophages (2×10^5/well) were incubated with varying concentrations ($1–10 \times 10^7$ particles/ml) of the glucan-rich *P. carinii* cell isolate for 12 h (∗, $p < 0.001$ compared with unstimulated macrophages). Each data point represents the mean ± SD of one representative experiment performed in triplicate.

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.
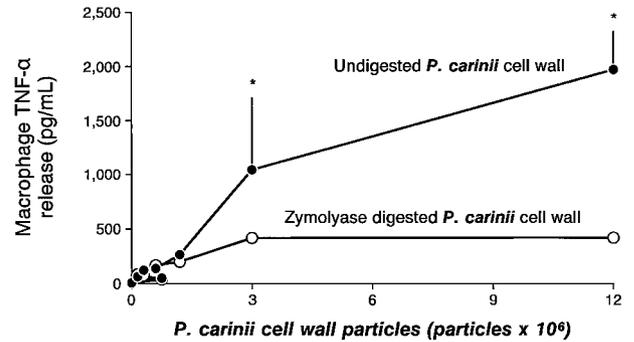

FIGURE 4. Digestion of *P. carinii* cell wall isolate with zymolyase inhibits its ability to activate macrophage TNF-α release. Alveolar macrophages were incubated with varying concentrations of zymolyase-digested *P. carinii* cell wall fraction ($0.75–25 \times 10^6$ particles/ml). As controls, macrophages were also stimulated with identical concentrations of undigested *P. carinii* cell wall particles. Digestion of the *P. carinii* cell wall isolate reduced the maximal observed TNF-α response from alveolar macrophages (∗, $p < 0.05$ comparing digested and undigested cell wall preparations). Each data point represents the mean ± SD of two experiments performed in duplicate.

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 ±
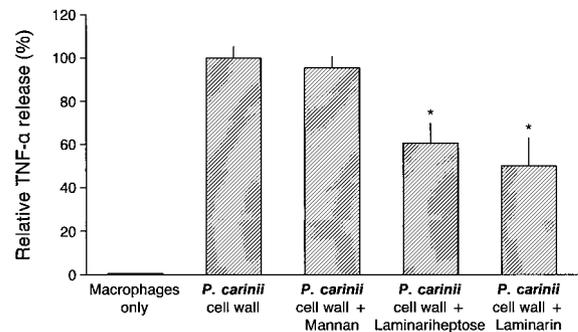

FIGURE 5. Soluble β-glucan but not mannose receptor antagonists suppress alveolar macrophage TNF-α release in response to *P. carinii* cell wall isolate. Incubation of alveolar macrophages with the soluble mannose receptor antagonist, α-mannan (0.5 mg/ml), did not significantly reduce the ability of *P. carinii* cell wall isolate to induce macrophage TNF-α release. In contrast, incubation of alveolar macrophages with the soluble β-glucan receptor antagonists, laminarin and laminariheptaose (0.5 mg/ml each), substantially decreased the observed TNF-α release induced by *P carinii* cell wall components (∗, $p < 0.05$ compared with control without antagonist). Each data point represents the mean ± SD of three experiments performed in triplicate.
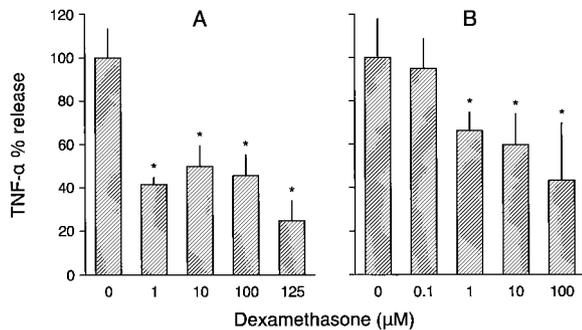
FIGURE 6. Dexamethasone reduces TNF-α release from alveolar macrophages stimulated with *P. carinii* cell wall isolate. *A*, Alveolar macrophages were incubated overnight with ~5 × 10^6 particles/ml of the *P. carinii* cell wall preparation in the presence of increasing concentrations of dexamethasone. The following day, TNF-α release into the medium was assessed using a L929 bioassay. Concentrations of dexamethasone equal to or greater than 1 μM significantly decreased TNF-α secretion (∗, $p < 0.05$ compared with macrophages in the absence of dexamethasone). *B*, Similar findings were observed when measurement of TNF-α was performed using an ELISA, again showing similar reduction of TNF-α release when macrophages were incubated with concentrations of dexamethasone ≥ 1 μM (∗, $p < 0.05$ compared with macrophages in the absence of dexamethasone). Each data point represents the mean ± SD of three experiments performed in triplicate.
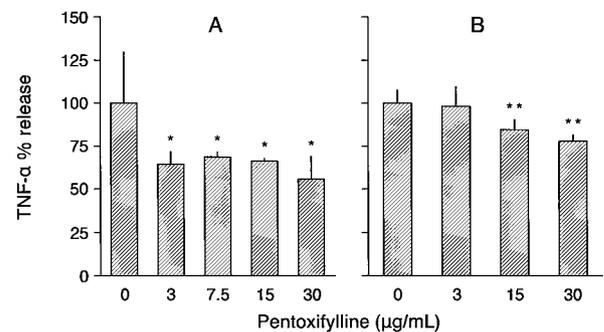

FIGURE 7. Pentoxifylline suppresses *P. carinii* cell wall-induced TNF-α release from alveolar macrophages. *A*, Alveolar macrophages were incubated overnight with ~5 × 10^6 particles/ml of *P. carinii* cell wall isolate in the presence of increasing concentrations of pentoxifylline. The following day, TNF-α release into the medium was assessed using a L929 bioassay. Concentrations of pentoxifylline equal to or greater than 15 μg/ml significantly decreased TNF-α secretion (∗, $p < 0.05$ compared with macrophages in the absence of pentoxifylline). *B*, Similar findings were observed when measurement of the TNF-α was performed using an ELISA, again showing similar reduction of TNF-α release when macrophages were incubated with concentrations of pentoxifylline = 15 μg/ml (∗∗, $p < 0.05$ compared with alveolar macrophages in the absence of pentoxifylline). Each data point represents the mean ± SD of three experiments performed in triplicate.

4.8% ($p = 0.0001$ compared with the alveolar macrophages stimulated with *P. carinii* cell wall isolate alone). Similarly, TNF-α release was also suppressed with the heptaglucosaccharide laminariheptaose by 37.8 ± 9.1% ($p = 0.035$ compared with control). In contrast, incubation of macrophages with yeast α-mannan, which antagonizes macrophage mannose receptors, did not cause any significant suppression of macrophage TNF-α release induced by the *P. carinii* cell wall isolate ($p = 0.36$ compared with macrophages incubated without α-mannan; Fig. 5). Therefore, these data strongly indicate that macrophage release of TNF-α following interaction with *P. carinii* cell wall predominantly occurs through interactions with macrophage β-glucan receptors.

It is highly unlikely that laminarin or laminariheptaose resulted in a nonspecific down-regulation of macrophage ability to secrete TNF-α. 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-α 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-α release from alveolar macrophages stimulated with the glucan-rich P. carinii *cell wall*

Attenuation of the TNF-α 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-α responses to the *P. carinii* cell wall isolate in vitro. Concentrations of dexamethasone equal to, or greater than, 1 μM resulted in suppression of the maximal observed TNF-α release by 43.8 ± 8.4% (Fig. 6; $p = 0.0001$ compared with the TNF-α release in the absence of dexamethasone). No significant difference was observed

whether TNF-α was assayed using either the L929 bioassay or ELISA. Maximum inhibition was observed with a dexamethasone concentration of 125 μM. Alveolar macrophages incubated with 125 μM dexamethasone before the addition of the *P. carinii* cell wall isolate released 66.7 ± 5.9% less TNF-α (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 (≥15 μg/ml) also resulted in significant suppression of macrophage TNF-α release induced by the *P. carinii* cell wall isolate. TNF-α release by the *P. carinii* cell wall was reduced by 34 ± 1.7% when alveolar macrophages were incubated with 15 μg/ml of pentoxifylline (Fig. 7; $p = 0.022$ compared with macrophages incubated without pentoxifylline). Maximal TNF-α suppression was observed with 30 μg/ml of pentoxifylline, which decreased the maximal observed TNF-α release by 44 ± 13% ($p = 0.0013$ compared with macrophages incubated in the absence of pentoxifylline). A similar response was observed whether TNF-α was measured with L929 or by ELISA (Fig. 7).

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-α 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-α-inflammatory response induced by the *P. carinii* cell wall is mediated principally by β-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-glucans. 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 isolate 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.

Acknowledgments

References

1. Limper, A. H., K. P. Offord, T. F. Smith, and W. J. d. Martin. 1989. *Pneumocystis carinii* pneumonia: differences in lung parasite number and inflammation in patients with and without AIDS. *Am. Rev. Respir. Dis. 140:1204.*

2. Yale, S. H., and A. H. Limper. 1996. *Pneumocystis carinii* pneumonia in patients without acquired immunodeficiency syndrome: associated illness and prior corticosteroid therapy. *Mayo Clin. Proc. 71:5.*

3. Mason, G. R., C. H. Hashimoto, P. S. Dickman, L. F. Foutty, and C. J. Cobb. 1989. Prognostic implications of bronchoalveolar lavage neutrophilia in patients with *Pneumocystis carinii* pneumonia and AIDS. *Am. Rev. Respir. Dis. 139:1336.*

4. Wright, T. W., C. J. Johnston, A. G. Harmsen, and J. N. Finkelstein. 1999. Chemokine gene expression during *Pneumocystis carinii*-driven pulmonary inflammation. *Infect. Immun. 67:3452.*

5. Smith, R. L., W. M. el-Sadr, and M. L. Lewis. 1988. Correlation of bronchoalveolar lavage cell populations with clinical severity of *Pneumocystis carinii* pneumonia. *Chest 93:60.*

6. Edman, J. C., J. A. Kovacs, H. Masur, D. V. Santi, H. J. Elwood, and M. L. Sogin. 1988. Ribosomal RNA sequence shows *Pneumocystis carinii* to be a member of the fungi. *Nature 334:519.*

7. Matsumoto, Y., S. Matsuda, and T. Tegoshi. 1989. Yeast glucan in the cyst wall of *Pneumocystis carinii*. *J. Protozool. 36:21S.*

8. Shematek, E. M., J. A. Braatz, and E. Cabib. 1980. Biosynthesis of the yeast cell wall. I. Preparation and properties of β-(1 leads to 3)glucan synthetase. *J. Biol. Chem. 255:888.*

9. Duffus, J. H., C. Levi, and D. J. Manners. 1982. Yeast cell-wall glucans. *Adv. Microb. Physiol. 23:151.*

10. Abel, G., and J. K. Czop. 1992. Stimulation of human monocyte β-glucan receptors by glucan particles induces production of TNF-α and IL-1β. *Int. J. Immunopharmacol. 14:1363.*

11. Castro, M., T. I. Morgenthaler, O. A. Hoffman, J. E. Standing, M. S. Rohrbach, and A. H. Limper. 1993. *Pneumocystis carinii* induces the release of arachidonic acid and its metabolites from alveolar macrophages. *Am. J. Respir. Cell Mol. Biol. 9:73.*

12. Olson, E. J., J. E. Standing, N. Griego-Harper, O. A. Hoffman, and A. H. Limper. 1996. Fungal β-glucan interacts with vitronectin and stimulates tumor necrosis factor α release from macrophages. *Infect. Immun. 64:3548.*

13. Rudmann, D. G., A. M. Preston, M. W. Moore, and J. M. Beck. 1998. Susceptibility to *Pneumocystis carinii* in mice is dependent on simultaneous deletion of IFN-γ and type 1 and 2 TNF receptor genes. *J. Immunol. 161:360.*

14. Chen, W., E. A. Havell, and A. G. Harmsen. 1992. Importance of endogenous tumor necrosis factor α and γ interferon in host resistance against *Pneumocystis carinii* infection. *Infect. Immun. 60:1279.*

15. Kolls, J. K., D. Lei, C. Vazquez, G. Odom, W. R. Summer, S. Nelson, and J. Shellito. 1997. Exacerbation of murine *Pneumocystis carinii* infection by adenoviral-mediated gene transfer of a TNF inhibitor. *Am. J. Respir. Cell Mol. Biol. 16:112.*

16. Yu, M. L., and A. H. Limper. 1997. *Pneumocystis carinii* induces ICAM-1 expression in lung epithelial cells through a TNF-α-mediated mechanism. *Am. J. Physiol. 273:L1103.*

17. Ward, P. A. 1998. Thematic review series 1: lung biology of chemokines and cytokines: introduction. *Proc. Assoc. Am. Physicians 110:287.*

18. Bozic, C. R., N. P. Gerard, C. von Uexkull-Guldenband, L. F. Kolakowski, Jr., M. J. Conklyn, R. Breslow, H. J. Showell, and C. Gerard. 1994. The murine interleukin 8 type B receptor homologue and its ligands: expression and biological characterization. *J. Biol. Chem. 269:29355.*

19. Bozzette, S. A., F. R. Sattler, J. Chiu, A. W. Wu, D. Gluckstein, C. Kemper, A. Bartok, J. Niosi, I. Abramson, J. Coffman, et al. 1990. A controlled trial of early adjunctive treatment with corticosteroids for *Pneumocystis carinii* pneumonia in the acquired immunodeficiency syndrome: California Collaborative Treatment Group. *N. Engl. J. Med. 323:1451.*

20. The National Institutes of Health-University of California Expert Panel for Corticosteroids as Adjunctive Therapy for Pneumocystis Pneumonia. 1990. Consensus statement on the use of corticosteroids as adjunctive therapy for pneumocystis pneumonia in the acquired immunodeficiency syndrome. *N. Engl. J. Med. 323: 1500.*

21. Refsum, S. E., M. I. Halliday, G. Campbell, M. McCaigue, B. J. Rowlands, and V. E. Boston. 1996. Modulation of TNF α and IL-6 in a peritonitis model using pentoxifylline. *J. Pediatr. Surg. 31:928.*

22. Sampaio, E. P., M. O. Moraes, J. A. Nery, A. R. Santos, H. C. Matos, and E. N. Sarno. 1998. Pentoxifylline decreases in vivo and in vitro tumor necrosis factor-α (TNF-α) production in lepromatous leprosy patients with erythema nodosum leprosum (ENL). *Clin. Exp. Immunol. 111:300.*

23. van Furth, A. M., E. M. Verhard-Seijmonsbergen, R. van Furth, and J. A. Langermans. 1997. Effect of lisofylline and pentoxifylline on the bacterial-stimulated production of TNF-α, IL-1β, IL-10 by human leukocytes. *Immunology 91:193.*

24. Hoffman, O. A., J. E. Standing, and A. H. Limper. 1993. *Pneumocystis carinii* stimulates tumor necrosis factor-α release from alveolar macrophages through a β-glucan-mediated mechanism. *J. Immunol. 150:3932.*

25. Tamburrini, E., A. De Luca, G. Ventura, G. Maiuro, A. Siracusano, E. Ortona, and A. Antinori. 1990. Macrophages from healthy adults release TNF-α after exposure to *Pneumocystis carinii* of murine origin: preliminary study. *Medicina 10:393.*

26. Limper, A. H., J. S. Hoyte, and J. E. Standing. 1997. The role of alveolar macrophages in *Pneumocystis carinii* degradation and clearance from the lung. *J. Clin. Invest. 99:2110.*

27. Limper, A. H., M. Edens, R. A. Anders, and E. B. Leof. 1998. *Pneumocystis carinii* inhibits cyclin-dependent kinase activity in lung epithelial cells. *J. Clin. Invest. 101:1148.*

28. Thomas, C. F., R. A. Anders, M. P. Gustafson, E. B. Leof, and A. H. Limper. 1998. *Pneumocystis carinii* contains a functional cell-division-cycle Cdc2 homologue. *Am. J. Respir. Cell Mol. Biol. 18:297.*

29. Figueiredo, F., L. M. Alves, and C. L. Silva. 1993. Tumor necrosis factor production in vivo and in vitro in response to *Paracoccidioides brasiliensis* and the cell wall fractions thereof. *Clin. Exp. Immunol. 93:189.*

30. Silva, C. L., L. M. Alves, and F. Figueiredo. 1994. Involvement of cell wall glucans in the genesis and persistence of the inflammatory reaction caused by the fungus *Paracoccidioides brasiliensis*. *Microbiology 140:1189.*

31. Bacon, J. S., V. C. Farmer, D. Jones, and I. F. Taylor. 1969. The glucan components of the cell wall of baker's yeast (*Saccharomyces cerevisiae*) considered in relation to its ultrastructure. *Biochem. J. 114:557.*

32. Kennedy, J. F., and G. Pagliuca. 1994. In *Carbohydrate Analysis*. M. F. Chaplin and J. F. Kennedy, eds. Oxford University Press, Oxford, p. 44.

33. Limper, A. H., U. Specks, W. M. Brutinel, W. J. d. Martin, and M. S. Rohrbach. 1993. Interlobar variation in the recovery of bronchoalveolar lavage fluid, cell populations, and angiotensin-converting enzyme in normal volunteers. *J. Lab. Clin. Med. 121:785.*

34. Castro, M., N. V. Ralston, T. I. Morgenthaler, M. S. Rohrbach, and A. H. Limper. 1994. *Candida albicans* stimulates arachidonic acid liberation from alveolar macrophages through α-mannan and β-glucan cell wall components. *Infect. Immun. 62:3138.*

35. Kramer, S. M., and M. E. Carver. 1986. Serum-free in vitro bioassay for the detection of tumor necrosis factor. *J. Immunol. Methods 93:201.*

36. Turtinen, L. W., and B. D. Juran. 1998. Standardization and comparison of an XTT-based TNF-α bioassay with a TNF-α ELISA. *BioTechniques 24:232.*

37. Neese, L. W., J. E. Standing, E. J. Olson, M. Castro, and A. H. Limper. 1994. Vitronectin, fibronectin, and gp120 antibody enhance macrophage release of TNF-α in response to *Pneumocystis carinii*. *J. Immunol. 152:4549.*

38. Czop, J. K., and J. Kay. 1991. Isolation and characterization of β-glucan receptors on human mononuclear phagocytes. *J. Exp. Med. 173:1511.*

39. Giaimis, J., Y. Lombard, P. Fonteneau, C. D. Muller, R. Levy, M. Makaya-Kumba, J. Lazdins, and P. Poindron. 1993. Both mannose and β-glucan receptors are involved in phagocytosis of unopsonized, heat-killed *Saccharomyces cerevisiae* by murine macrophages. *J. Leukocyte Biol. 54:564.*

40. O'Riordan, D. M., J. E. Standing, and A. H. Limper. 1995. *Pneumocystis carinii* glycoprotein A binds macrophage mannose receptors. *Infect. Immun. 63:779.*

41. Millar, A. B., R. F. Miller, N. M. Foley, A. Meager, S. J. Semple, and G. A. Rook. 1991. Production of tumor necrosis factor-α by blood and lung mononuclear phagocytes from patients with human immunodeficiency virus-related lung disease. *Am. J. Respir. Cell Mol. Biol. 5:144.*

42. Vilcek, J., and T. H. Lee. 1991. Tumor necrosis factor: new insights into the molecular mechanisms of its multiple actions. *J. Biol. Chem. 266:7313.*

43. Pesanti, E. L., T. Tomicic, and S. T. Donta. 1991. Binding of ^{125}I-labelled tumor necrosis factor to *Pneumocystis carinii* and an insoluble cell wall fraction. *J. Protozool. 38:28S.*

44. Pesanti, E. L. 1991. Interaction of cytokines and alveolar cells with *Pneumocystis carinii* in vitro. *J. Infect. Dis. 163:611.*

45. Hyers, T. M., S. M. Tricomi, P. A. Dettenmeier, and A. A. Fowler. 1991. Tumor necrosis factor levels in serum and bronchoalveolar lavage fluid of patients with the adult respiratory distress syndrome. *Am. Rev. Respir. Dis. 144:268.*

46. Jaattela, M. 1991. Biologic activities and mechanisms of action of tumor necrosis factor-α/cachectin. *Lab. Invest. 64:724.*

47. Gagnon, S., A. M. Boota, M. A. Fischl, H. Baier, O. W. Kirksey, and L. La Voie. 1990. Corticosteroids as adjunctive therapy for severe *Pneumocystis carinii* pneumonia in the acquired immunodeficiency syndrome: a double-blind, placebo-controlled trial. *N. Engl. J. Med. 323:1444.*

48. Kullmann, A., P. Vaillant, V. Muller, Y. Martinet, and N. Martinet. 1993. In vitro effects of pentoxifylline on smooth muscle cell migration and blood monocyte production of chemotactic activity for smooth muscle cells: potential therapeutic benefit in the adult respiratory distress syndrome. *Am. J. Respir. Cell Mol. Biol. 8:83.*

49. Sheridan, B. C., R. C. McIntyre, D. R. Meldrum, and D. A. Fullerton. 1997. Pentoxifylline treatment attenuates pulmonary vasomotor dysfunction in acute lung injury. *J. Surg. Res. 71:150.*

50. Kolls, J. K., J. M. Beck, S. Nelson, W. R. Summer, and J. Shellito. 1993. Alveolar macrophage release of tumor necrosis factor during murine *Pneumocystis carinii* pneumonia. *Am. J. Respir. Cell Mol. Biol. 8:370.*

51. Corsini, E., C. Dykstra, W. A. Craig, R. R. Tidwell, and G. J. Rosenthal. 1992. *Pneumocystis carinii* induction of tumor necrosis factor-α by alveolar macrophages: modulation by pentamidine isethionate. *Immunol. Lett. 34:303.*

52. Ezekowitz, R. A., D. J. Williams, H. Koziel, M. Y. Armstrong, A. Warner, F. F. Richards, and R. M. Rose. 1991. Uptake of *Pneumocystis carinii* mediated by the macrophage mannose receptor. *Nature 351:155.*

53. Hidalgo, H. A., R. J. Helmke, V. F. German, and J. A. Mangos. 1991. Role of the zymolyase sensitive cell wall of *Pneumocystis carinii* in the oxidative burst of macrophages. *J. Protozool. 38:38S.*

54. Okazaki, M., Y. Adachi, N. Ohno, and T. Yadomae. 1995. Structure-activity relationship of (1→3)-β-D-glucans in the induction of cytokine production from macrophages, in vitro. *Biol. Pharm. Bull. 18:1320.*

55. Michalek, M., D. Melican, D. Brunke-Reese, M. Langevin, K. Lemerise, W. Galbraith, M. Patchen, and W. Mackin. 1998. Activation of rat macrophages by Betafectin PGG-glucan requires cross-linking of membrane receptors distinct from complement receptor three (CR3). *J. Leukocyte Biol. 64:337.*