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Fungal β-Glucan Interacts with Vitronectin and Stimulates Tumor Necrosis Factor Alpha Release from Macrophages

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β-Glucans are polymers of D-glucose which represent major structural components of fungal cell walls. It was shown previously that fungi interact with macrophages through β-glucan receptors, thereby inducing release of tumor necrosis factor alpha (TNF-α). Additional studies demonstrated that vitronectin, a host adhesive glycoprotein, binds to fungi and enhances macrophage recognition of these organisms. Since vitronectin contains a carbohydrate-binding region, we postulated that vitronectin binds fungal β-glucans and subsequently augments macrophage TNF-α release in response to this fungal component. To study this, we first determined the release of TNF-α from alveolar macrophages stimulated with fungal β-glucan. Maximal TNF-α release occurred with moderate concentrations of β-glucan (100 to 200 μg/ml), whereas higher concentrations of β-glucan (≥500 μg/ml) caused apparent suppression of the TNF-α activity released. This suppression of TNF-α activity by high concentrations of β-glucan was 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. Next, we assessed the interaction of vitronectin with β-glucan. Binding of ^{125}I-vitronectin to particulate fungal β-glucan was dose dependent and specifically inhibitable by unlabeled vitronectin. Furthermore, treatment of β-glucan with vitronectin substantially augmented macrophage TNF-α release in response to this fungal component. These findings demonstrate that fungal β-glucan can directly modulate TNF-α release from macrophages. Further, these studies indicate that the host adhesive glycoprotein vitronectin specifically binds β-glucan and augments macrophage cytokine release in response to this fungal element.

Alveolar macrophages are important mediators of host defense against pathogenic fungi infecting the lungs, such as *Pneumocystis carinii*, *Cryptococcus neoformans*, *Aspergillus fumigatus*, *Histoplasma capsulatum*, and *Candida albicans* (5, 9, 11, 30, 35, 37, 57). Upon interaction with fungal cell surface components, macrophages bind and phagocytize fungal organisms and are stimulated to release inflammatory mediators, including tumor necrosis factor alpha (TNF-α) (3, 6, 10, 14). TNF-α plays a crucial role in host defense against fungi by stimulating the release of other inflammatory cytokines, enhancing vascular endothelial cell permeability, promoting recruitment of additional immune effector cells, and activating neutrophil killing of fungal organisms (15, 17, 28). In addition, TNF-α has been proposed to directly affect the viability of certain organisms, such as *P. carinii* and trypanosomes (32, 42, 43). The mechanisms by which macrophages recognize fungi, thereby inducing the release of TNF-α, have only recently been investigated (6, 13, 14).

Current studies indicate that macrophages release TNF-α following interaction of membrane β-glucan receptors with fungal organisms (13, 14). β-Glucans are conserved structural elements of most fungal cell walls, composed of D-glucose polymers constructed primarily through β-1,3 and β-1,6 linkages (34). Previous studies indicated that fungal β-glucans induce significant TNF-α release from macrophages in response to both *P. carinii* and *C. albicans* (3, 14). However, fungal β-glucans also possess immunomodulatory activity. Additional work from our laboratory indicates that β-glucans at selective doses can either induce or suppress the release of TNF-α from mononuclear phagocytes (13). Whereas low and intermediate concentrations of β-glucans strongly induce TNF-α release, high concentrations of β-glucans (≥500 μg/ml) suppress liberation of TNF-α from rodent macrophages (13). Furthermore, when macrophages are cultured with higher concentrations of β-glucans, they are refractory to TNF-α release even in response to lipopolysaccharide (LPS), a potent independent stimulant of TNF-α generation (13). Other investigators have shown that treatment of animals with derivatized β-glucans prior to gram-negative bacterial infection of the peritoneal cavity blunts circulating TNF-α levels and prevents death from sepsis (44, 48). The mechanisms by which β-glucans can mediate these apparently contrasting effects on cytokine activation are not yet fully understood.

Recent studies also indicate that certain host adhesive proteins further modulate the interaction of fungal β-glucans with mononuclear phagocytes and macrophages and subsequently alter cytokine release from phagocytes in response to fungal organisms (29, 36, 38). In particular, we have determined that vitronectin, a 75-kDa host adhesive glycoprotein present in the circulation and in the alveolar spaces, significantly potentiates macrophage recognition of *P. carinii* and enhances TNF-α release in response to the organism (31, 36). Additional studies reveal that vitronectin also interacts specifically with *C. albicans* and promotes attachment of this organism to cultured NR8383 macrophage cells (30).

Vitronectin contains a number of domains capable of interacting with microorganisms and mammalian cells, including an Arg-Gly-Asp sequence which binds eukaryotic integrin receptors and a glycosaminoglycan-binding domain which is capable

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of interacting with complex carbohydrates and glycoconjugates (12, 52). Vitronectin's glycosaminoglycan-binding domain interacts with a high-molecular-weight component on *P. carinii*, consistent with a fungal β-glucan (31). Similarly, the binding of vitronectin to *C. albicans* also maps to the glycosaminoglycan-binding domain of the molecule, again suggesting that vitronectin recognizes a glycoconjugate on the surface of this fungus as well (30). On the basis of these observations, we hypothesized that vitronectin binds to β-glucans present in fungal cell walls and subsequently augments macrophage recognition and TNF-α release in response to the organism. As such, the interaction of vitronectin with fungal β-glucans represents a generalized mechanism of host interaction with fungi.

To test these hypotheses, we evaluated the interaction of vitronectin with β-glucan derived from the common yeast *Saccharomyces cerevisiae* and determined whether the interaction altered macrophage cytokine release in response to this fungal cell wall component. This study was designed specifically to (i) define the concentration-response characteristics of TNF-α release from rabbit alveolar macrophages challenged with fungal β-glucan, (ii) determine potential mechanisms by which β-glucans can both induce and suppress TNF-α release, (iii) evaluate the extent to which vitronectin binds specifically to fungal β-glucan particles, and (iv) determine whether the interaction of vitronectin with fungal β-glucan increases macrophage release of TNF-α in response to this fungal element.

MATERIALS AND METHODS

Materials. All organic chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.) unless otherwise specified. Iodo-Beads were obtained from Pierce Chemical Co. (Rockford, Ill.), and carrier-free $Na^{125}I$ and ^{125}I-labeled recombinant human TNF-α were from Amersham (Arlington Heights, Ill.). Fungal β-glucan, derived from *S. cerevisiae*, having a mean particle size of 4.2×10^{10} glucose residues, was obtained from Sigma Chemical Co. These β-glucan preparations contained <0.125 U of soluble endotoxin per ml as assessed by a sensitive *Limulus* amebocyte lysate assay (13). Recombinant human and murine TNF-α was obtained from Amersham and Genzyme Corporation (Cambridge, Mass.), respectively.

Stimulation of rabbit alveolar macrophages with particulate fungal β-glucan. We first determined the concentration-response characteristics of TNF-α release from rabbit alveolar macrophages challenged with fungal β-glucan. Alveolar macrophages were obtained from New Zealand White rabbits by lavage with Hanks balanced salt solution (HBSS) (200 ml) as described previously (4). The lavage was centrifuged (400 $g \times 10$ min) and washed with HBSS, and the cells were resuspended in mixed medium (equal volumes of medium 199 and RPMI 1640) supplemented with 2 mM glutamine, 10,000 U of penicillin/liter, 1 mg of streptomycin/liter, and 25 μg of amphotericin per ml. The cultures typically contained >95% macrophages (4). The macrophages were plated on 96-well plates (200,000 cells per well), incubated for 1 h at 37°C, and washed to remove nonadherent cells. Prior studies indicate that >95% of the macrophages were adherent after this initial incubation (4, 5). Fungal β-glucan particles were suspended in mixed medium by probe sonication and added to the cells at the concentrations indicated below (0 to 1,000 μg/ml). After overnight incubation, the medium was removed, clarified by centrifugation (10,000 $g \times 4$ min), and assayed for TNF-α content.

Determination of TNF-α release from macrophages. TNF-α content in the medium was determined by a modified L929 cytotoxicity assay (14, 16, 24). Murine L929 monolayers were grown to confluence in Eagle's minimum essential medium containing 5% fetal calf serum, harvested with trypsin (1:250 with EDTA), and plated on 96-well plates (4.0×10^5 per well) overnight at 37°C. The following day, the medium was replaced with Eagle's minimum essential medium containing actinomycin D (1 μg/ml). Since rabbit TNF-α was not commercially available, recombinant murine TNF-α (Genzyme) was used as a relative standard of bioactivity for these assays. Standards or samples were plated onto the L929 cells, and the cells were incubated for 18 h at 37°C. Thereafter, the medium was removed, the cells were stained with 0.5% crystal violet, and the A_{490}s were determined. A standard concentration curve was prepared by using the optical densities of the standards and was used to calculate the TNF-α contents of the samples. Since the absolute quantities of TNF-α are based upon a relative standard of murine TNF-α bioactivity, the data were normalized so that TNF-α levels were expressed as percent maximal TNF-α release, in which the TNF-α released in stimulations conducted with 200 μg of β-glucan per ml was defined as 100%. In all cases, the TNF-α activity measurements were within the linear range of responsiveness of L929 cells to TNF-α in these assays.

Determination of TNF-α binding to fungal β-glucan particles. We postulated that the apparent suppression of TNF-α activity resulting from higher concentrations of β-glucan (≥500 μg/ml) was in part mediated by soluble TNF-α binding to the insoluble β-glucan particles. To address this, the binding of radiolabeled TNF-α to suspended β-glucan particles was examined. Particulate β-glucan in HBSS (1,000 μg/ml) was incubated with ^{125}I–TNF-α (0 to 50 ng/ml) in Tris-buffered saline (TBS) containing calcium (1 mM) and bovine serum albumin (BSA) (1 mg/ml) at 37°C for 60 min. The β-glucan particles containing bound ^{125}I–TNF-α were separated from free ligand by centrifugation through oil (dibutyl phthalate-apezion oil, 9:1), and binding of ^{125}I–TNF-α was determined by gamma counting. To measure nonspecific binding, assays were also conducted in the presence of unlabeled TNF-α (1 μg/ml). Additional TNF-α binding studies were also conducted in the presence of the competitive carbohydrates *N*,*N'*-diacetylchitobiose, D-cellobiose, D-glucose, and L-fucose to assess interaction of fungal β-glucan with the lectin-binding region of TNF-α.

Purification and characterization of vitronectin. Vitronectin was purified from human plasma by heparin affinity chromatography as previously reported (31, 58). In brief, outdated human plasma, obtained from the Mayo Blood Products Laboratory, was clotted by the addition of calcium (final concentration, 20 mM), the plasma-derived serum was collected following centrifugation, and phenylmethylsulfonyl fluoride (1 mM) and EDTA (5 mM) were added. A heparin-Sepharose column (10-ml bed volume) was equilibrated with 10 mM sodium phosphate containing 5 mM EDTA (pH 7.7) and preabsorbed with the plasma-derived serum. The heparin-binding activity of the serum was next activated by addition of urea (8 mM), permitting the vitronectin to bind to the heparin-Sepharose column. The urea-activated serum was again passed over the column. Following a washing with 10 mM sodium phosphate containing 5 mM EDTA, the column was eluted with 500 mM NaCl in 8 mM urea to yield the purified vitronectin. The recovered fractions were exhaustively dialyzed against TBS. These preparations were assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver staining to be >95% pure. The identity of vitronectin was confirmed by amino-terminal sequencing (31). These vitronectin isolates contained <0.125 U of endotoxin per ml (36). Purified vitronectin (1.0 mg in 0.5 ml of phosphate-buffered saline) was iodinated with 1 mCi of $Na^{125}I$ in a siliconized glass tube for 10 min at 20°C with four Iodo-Beads, yielding a specific activity of approximately 200,000 cpm/μg.

Interaction of vitronectin with particulate fungal β-glucan. Increasing concentrations of ^{125}I-labeled vitronectin were incubated for 1 h with β-glucan particles (1,000 μg/ml) suspended in TBS with 1 mg of BSA per ml at 25°C. Following incubation, the β-glucan particles containing bound ^{125}I-vitronectin were separated from free ligand by centrifugation through oil (dibutyl phthalate-apezion oil, 9:1), and bound vitronectin was determined by gamma counting. To evaluate nonspecific binding, assays were also performed with addition of a 100-fold excess of unlabeled vitronectin.

Effect of vitronectin on TNF-α release from macrophages stimulated with fungal β-glucan. Prior investigations indicated that vitronectin bound to the surface of fungi enhances macrophage release of TNF-α (36). Therefore, we next determined whether vitronectin binding to β-glucan particles similarly enhances macrophage release of TNF-α in response to this specific fungal cell wall component. Accordingly, β-glucan particles were incubated with either vitronectin (100 μg/ml) or HBSS (control) at 37°C for 30 min. Previous studies have shown vitronectin to interact significantly with fungal organisms at comparable concentrations (30, 31, 36). After being coated with vitronectin, the β-glucan particles were washed with 1.5 ml of HBSS to remove nonbound protein and collected by centrifugation (14,000 $\times g$, 5 min). Such vitronectin treatment of fungal β-glucan particles evaluates only the effect of vitronectin which was bound to the β-glucan particles. Free or loosely associated vitronectin is therefore removed from the fungal β-glucan particles prior to stimulation of the macrophages (36). Vitronectin-coated β-glucan or noncoated control β-glucan particles were incubated overnight with macrophages at 37°C in mixed medium as indicated below. Subsequently, the medium was removed, and TNF-α release was determined.

Statistical analysis. Data are expressed as means ± standard errors of the means (SEM) from at least three determinations. Differences between datum groups were compared by using unpaired Student's *t* tests. Statistical testing was done with the Statview II statistical package (Abacus Concepts, Berkeley, Calif.). A P of ≤0.05 was defined as a statistically significant difference.

RESULTS

Intermediate concentrations of fungal β-glucan particles induce TNF-α release from rabbit alveolar macrophages. The fungal cell wall component β-glucan induced the release of TNF-α from rabbit alveolar macrophages in a biphasic dose-dependent manner (Fig. 1). Maximal TNF-α release from alveolar macrophages into the culture medium was observed after overnight incubation with 200 μg of fungal β-glucan particles per ml ($P = 0.0003$ compared with unstimulated macrophages). This maximal level of TNF-α release was defined as 100%. Macrophages cultured without β-glucan spontaneously released (11.4 ± 4.5)% (mean ± SEM) of this maximal level.

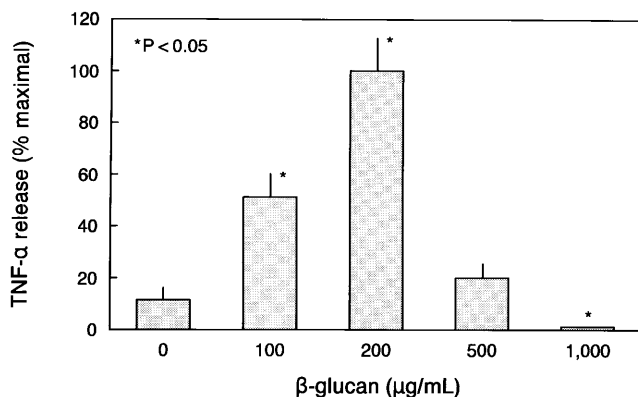

FIG. 1. Particulate fungal β-glucan induces the release of TNF-α from rabbit alveolar macrophages. Alveolar macrophages were obtained from rabbits by lavage and challenged with increasing concentrations of fungal β-glucan particles overnight. The following day, the media were removed, clarified, and assayed for TNF-α activity with an L929 cytotoxicity assay. Intermediate concentrations (100 to 200 μg/ml) of β-glucan stimulated TNF-α release, whereas higher concentrations (500 to 1000 μg/ml) caused suppressed release of TNF-α activity. Shown are means ± SEM of four determinations. *, $P < 0.05$ in comparison of β-glucan-stimulated macrophages with control macrophages cultured without fungal β-glucan.

In contrast, incubation with 100 μg of β-glucan particles per ml induced TNF-α release at (51.1 ± 8.7)% of the maximal level ($P = 0.013$ compared with unstimulated controls). Interestingly, higher concentrations of fungal β-glucan particles (≥500 μg/ml) consistently caused an apparent suppression of TNF-α release from the macrophages. Incubation with 500 μg of β-glucan per ml decreased TNF-α activity to near baseline levels [(20.1 ± 5.2)% maximal; $P = 0.077$ compared with unstimulated controls]. Further, when challenged with 1,000 μg of β-glucan per ml, alveolar macrophages yielded just (1.3 ± 0.3)% of maximal TNF-α release. It is noteworthy that the level of TNF-α activity detected in the medium following culture with 1,000 μg of β-glucan per ml was significantly below the level of TNF-α activity spontaneously released from alveolar macrophages cultured in the absence of β-glucan particles ($P = 0.0017$). These results demonstrate that β-glucan can both induce and suppress TNF-α release from rabbit alveolar macrophages. These data parallel our prior observations for β-glucan-challenged macrophages derived from rats (13).

Fungal β-glucan particles bind TNF-α through lectin-like interactions. As noted above, the level of measurable release of TNF-α activity from macrophages in the presence of high concentrations of β-glucan (1,000 μg/ml) was actually below the level of TNF-α spontaneously released from unstimulated macrophages. This suggested that in the face of high concentrations of fungal β-glucan (≥500 μg/ml), the excess nonphagocytized β-glucan particles might suppress TNF-α activity by binding up the cytokine, thus rendering it unavailable for measurement. Prior studies demonstrated that TNF-α exhibits lectin binding with certain glycoconjugates (32, 33, 49). To test this, the interaction of radiolabeled TNF-α with particulate fungal β-glucan was studied. Total TNF-α binding to β-glucan was examined by culturing β-glucan (1,000 μg/ml) with ^{125}I–TNF-α as described above (Fig. 2). Specific binding of TNF-α to the particulate β-glucan was determined in parallel by culturing ^{125}I–TNF-α at the indicated concentrations with β-glucan in the presence of excess, unlabeled human recombinant TNF-α (1 μg/ml). The difference between the total and the nonspecific binding indicates that significant specific binding of ^{125}I–TNF-α to the fungal β-glucan particles was detectable at concentrations of TNF-α of ≥10 ng/ml ($P \leq 0.05$ for comparison of total and nonspecific binding). With a higher concentration of TNF-α (50 ng/ml), we also observed a significant difference between total and nonspecific TNF-α binding to fungal β-glucan particles, indicating significant specific binding under these conditions as well (Fig. 2). However, the degree of nonspecific binding at these higher concentrations is greater than that observed at lower levels of TNF-α. This additional binding might represent ionic, additional lectin-type, or other binding interactions. However, even the TNF-α bound to fungal β-glucan particles through these alternate nonspecific mechanisms at higher concentrations is removed from solution, thereby reducing the TNF-α activity available in the medium.

To further test the ability of fungal β-glucan particles to bind TNF-α released from macrophages in response to an independent potent agonist, the following experiment was performed. Rabbit alveolar macrophages (200,000 per well) were incubated with LPS (5 μg/ml, from *Escherichia coli* O127:B8 [Sigma]) overnight. The following day, the medium was removed, incubated with β-glucan particles (1,000 μg/ml) for 1 h, and clarified by centrifugation. Incubation of the medium with β-glucan particles decreased the measurable TNF-α by (47.8 ± 8.9)% (mean ± SEM; $P = 0.028$). Taken together, these data indicate that excess nonphagocytized fungal β-glucan particles bind TNF-α released from alveolar macrophages.

We further sought to determine whether the binding of TNF-α to fungal β-glucan was mediated through the lectin-binding domain of the cytokine. To test this, TNF-α binding assays were conducted in the presence of increasing concentrations of *N,N'*-diacetylchitobiose, D-cellobiose, D-glucose, and L-fucose (Fig. 3). The binding of TNF-α to β-glucan was most effectively inhibited by *N,N'*-diacetylchitobiose (50% effective dose [ED_{50}] = 15 mM) (Fig. 3A). In contrast, D-cellobiose was ineffective in preventing binding of ^{125}I–TNF-α to fungal β-glucan particles (ED_{50}, >250 mM). We further observed that D-glucose, the principal sugar constituent of fungal β-glucan, could significantly inhibit TNF-α binding to fungal β-glucan, although it was not nearly as effective a competitor as *N,N'*-diacetylchitobiose. The ED_{50} for half-maximal inhibition of TNF-α binding with D-glucose was 500 mM (Fig. 3B). The specific binding of TNF-α to β-glucan was not altered by comparable concentrations of L-fucose (ED_{50}, >1,000 mM). A

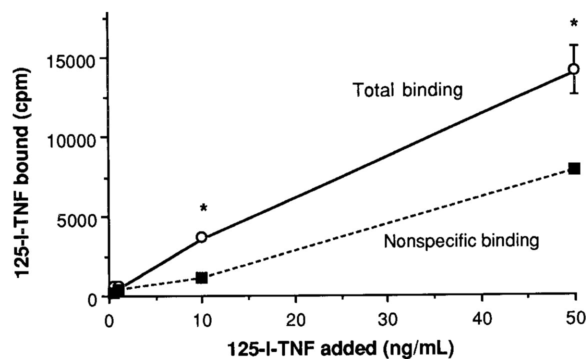

FIG. 2. TNF-α binds specifically to fungal β-glucan particles. Increasing concentrations of ^{125}I-labeled TNF-α were incubated with fungal β-glucan particles in the presence or absence of unlabeled TNF-α. Following separation of bound and free TNF-α, the bound ligand was evaluated by gamma counting. Total binding was considered ^{125}I–TNF-α bound in the absence of unlabeled ligand. In contrast, nonspecific binding was that remaining in the presence of excess unlabeled TNF-α. Each datum point is the mean ± SEM of three determinations. *, $P < 0.05$ in comparison of total and nonspecific binding.

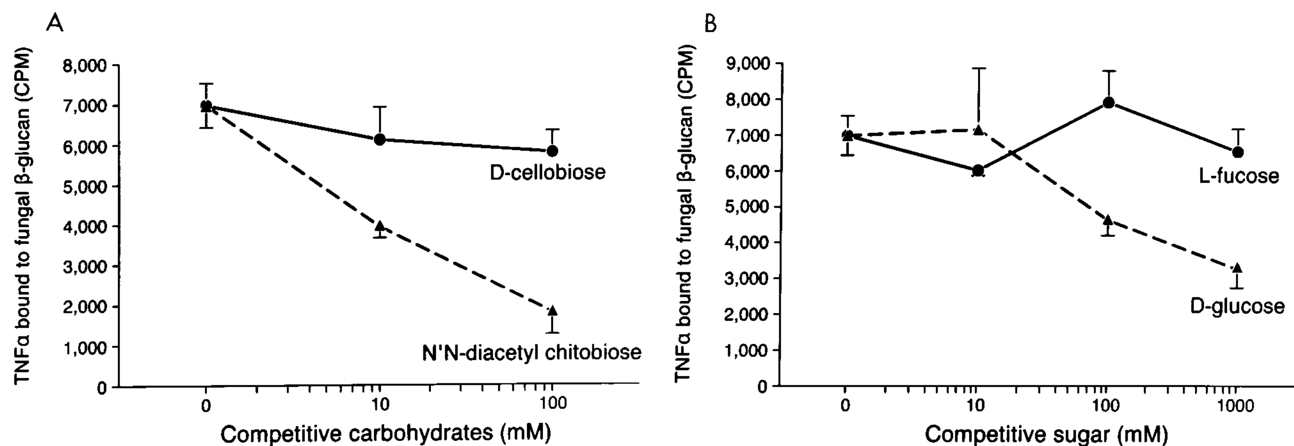

FIG. 3. TNF-α interacts with fungal β-glucan through lectin-mediated mechanisms. To further determine whether TNF-α binding to β-glucan occurred through the lectin-binding domain of the cytokine, additional TNF-α binding assays were performed in the presence of increasing concentrations of competitive sugar ligands. ^{125}I–TNF-α (10 ng/ml) was incubated with particulate β-glucan (1,000 μg/ml) and the indicated concentrations of competing sugars for 1 h at room temperature. Bound ^{125}I–TNF-α was separated from free TNF-α by centrifugation through oil. (A) *N,N'*-diacetylchitobiose but not D-cellobiose caused marked inhibition of TNF-α binding to β-glucan particles. (B) D-Glucose also caused significant reduction of TNF-α binding to β-glucan but required much larger concentrations of the sugar. L-Fucose had no effect on TNF-α binding. This pattern of sugar inhibition is consistent with fungal β-glucan particles interacting with the lectin-binding region of TNF-α. Shown are the means ± SEM for four determinations.

similar pattern of sugar inhibition has previously been reported for the lectin-mediated binding of TNF-α to the glycoprotein uromodulin (49). Taken together, these data indicate that fungal cell wall β-glucan can bind TNF-α through interaction with the lectin-binding domain of the cytokine.

Vitronectin interacts specifically with fungal β-glucan particles. Vitronectin is known to interact with fungi through its glycoaminoglycan-binding domain (30, 31). We therefore sought to determine whether vitronectin would interact specifically with fungal cell wall β-glucan. The binding of ^{125}I-vitronectin to β-glucan particles was assessed by suspension binding analysis (Fig. 4). ^{125}I-vitronectin exhibited dose-dependent binding to suspended β-glucan particles with increasing concentrations of the ligand. To assess for specificity of binding, ^{125}I-vitronectin binding assays were also conducted in the presence of 100-fold excess unlabeled vitronectin. Specific ligand binding is represented as the difference between total and nonspecific binding. Significant binding of vitronectin to β-glucan occurred with as little as 1.2 μg of the radiolabeled ligand per ml. Thus, vitronectin, a ubiquitous host adhesive protein, exhibited specific, dose-dependent interaction with fungal cell wall β-glucan.

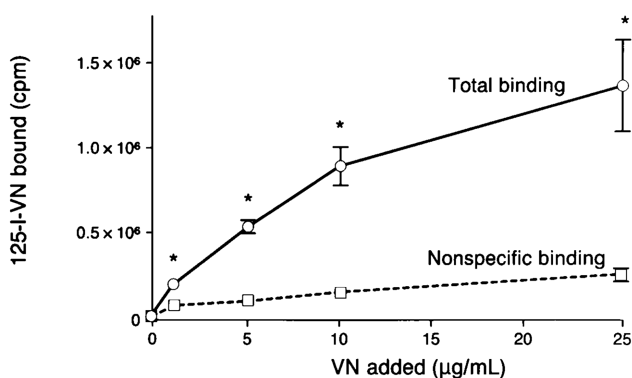

FIG. 4. The adhesive glycoprotein vitronectin binds fungal β-glucans. Increasing concentrations of ^{125}I-labeled vitronectin were incubated with fungal β-glucan particles in the presence or absence of excess unlabeled vitronectin. Following separation of bound and free vitronectin, the bound vitronectin was quantified by gamma counting. Total binding was the ^{125}I-vitronectin bound in the absence of unlabeled ligand. In contrast, nonspecific binding was represented as that binding remaining in the presence of 100-fold excess unlabeled vitronectin. The relative difference is a measure of specific binding. Significant specific binding was observed with as little as 1.2 μg of ^{125}I-vitronectin per ml. Each datum point represents the mean ± SEM of three determinations. *, $P < 0.05$ in comparison of total and nonspecific binding. VN, vitronectin.

Vitronectin binding to β-glucan augments macrophage release of TNF-α in response to the fungal component. Prior work in our laboratory indicated that interaction of vitronectin with intact fungal organisms enhances macrophage recognition and activation to release TNF-α (36). The current investigation demonstrates that vitronectin binds to β-glucan, a major structural component of fungal cell walls. We therefore questioned whether vitronectin coating of fungal β-glucan particles would similarly enhance macrophage TNF-α release in response to this fungal element. To address this, macrophage release of TNF-α was determined following stimulation with vitronectin-coated and -uncoated (control) particulate fungal β-glucan (Fig. 5). Vitronectin coating of fungal β-glucan particles resulted in significantly enhanced TNF-α release from alveolar macrophages. We observed that TNF-α release from macrophages stimulated with 100 μg of vitronectin-coated fungal β-glucan per ml was augmented to (367.0 ± 36.3)% (mean ± SEM) compared with that of macrophages stimulated with β-glucan particles not coated with vitronectin ($P = 0.001$). Thus, interaction of vitronectin with β-glucan enhances the macrophage cytokine response to this fungal component. Interestingly, however, at a high concentration of β-glucan particles (1,000 μg/ml), the level of TNF-α activity released was again suppressed to baseline, even following coating of the particles with vitronectin.

DISCUSSION

Recognition of β-glucans is an important mechanism of host interaction with fungal pathogens (6, 14, 19). Our current investigations demonstrate that while intermediate concentrations of fungal β-glucan particles stimulate release of TNF-α from macrophages, higher concentrations of β-glucan inhibit release of TNF-α activity from host cells. This β-glucan-medi-

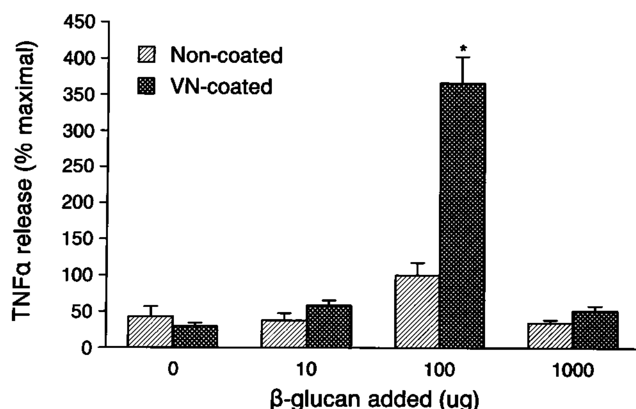

FIG. 5. Interaction of vitronectin with fungal β-glucan augments macrophage release of TNF-α. Increasing concentrations of β-glucan were incubated for 30 min with vitronectin (100 μg/ml) or HBSS alone (noncoated controls) and washed to remove nonbound protein. Noncoated and vitronectin-coated fungal β-glucan particles were then incubated overnight with rabbit alveolar macrophages, and TNF-α release was determined by L929 assay. Vitronectin coating substantially enhanced the release of TNF-α from macrophages stimulated with intermediate concentrations of β-glucan (100 μg/ml). Shown are means ± SEM of three determinations. *, $P = 0.001$ in comparison of vitronectin-coated glucan and noncoated controls. VN, vitronectin.

ated suppression of TNF-α activity occurs in part through β-glucan binding of TNF-α. We additionally demonstrate that vitronectin, a host adhesive protein, interacts with β-glucan and enhances macrophage TNF-α release in response to this fungal cell wall component.

β-Glucans are homopolymers of D-glucose, containing β-1,3 and β-1,6 linkages (34). These conserved structural elements are present in most yeast and fungal cell walls and occur in grains and other plant species. β-Glucans interact with 160- to 180-kDa β-glucan receptors present on peripheral blood monocytes and macrophages (1, 7, 18, 20, 53). The interactions of β-glucans with host cells upregulate host responses in a variety of infectious and neoplastic conditions, in part through activation of mononuclear phagocytes (21–23, 39–41, 45–47, 51, 54). Binding of β-glucans to cognate receptors on monocytes and macrophages participates in uptake of the fungal organisms *C. albicans*, *P. carinii*, and *C. neoformans* (6, 14, 19). Additionally, β-glucans initiate secretion of TNF-α from macrophages in response to *P. carinii* and eicosanoid generation by macrophages challenged with *C. albicans* (3, 4, 14). Other investigators have demonstrated that particulate β-glucans also induce the release of TNF-α and interleukin 1β from human peripheral blood monocytes and promote liberation of eicosanoids and lysozyme from these cells (7, 8, 18, 50).

Although many studies demonstrate that fungal β-glucans mediate inflammatory activation, emerging evidence suggests that β-glucans also possess immunosuppressive activity under selective circumstances. For instance, mice can be protected from lethal infection with *E. coli* by prior administration of β-glucans intraperitoneally (48). Animals treated with β-glucan do not exhibit the toxic elevations of TNF-α levels observed in untreated controls. Studies from our laboratory have further demonstrated that alveolar macrophages exposed to high concentrations of β-glucan release comparatively little TNF-α (13). Alveolar macrophages challenged with large quantities of fungal β-glucan (≥500 μg/ml) are refractory to further TNF-α release by potent agents such as LPS (13). The present study demonstrates that such concentrations of β-glucan mediate suppression of TNF-α by binding cytokine released from the phagocyte.

The binding of TNF-α to fungal β-glucan is mediated by the lectin-binding region of the cytokine. The interaction of TNF-α with trypanosomes and with uromodulin also occurs through this lectin-binding domain, which is functionally and spatially distinct from the region of the molecule which interacts with mammalian TNF-α receptors (32, 49). Lectin-binding interactions of TNF-α can be best inhibited with *N,N'*-diacetylchitobiose and likely occur through binding of the carbohydrate core rather than binding of terminal sugar residues of the ligand. In light of the substantial data supporting the protective benefits of β-glucan derivatives during experimental sepsis, further investigations to evaluate the potential utility of such TNF-α-binding strategies in states characterized by excessive production of TNF-α are indicated. β-Glucan derivatives might prove beneficial in conditions with acute elevations of TNF-α levels, such as sepsis and multiorgan failure, or in disorders with chronic elevations of TNF-α levels, such as malignancy or human immunodeficiency virus infection (2, 55, 56).

In contrast, however, we observed that low and intermediate concentrations of β-glucan (100 to 200 μg/ml) substantially stimulated TNF-α release from macrophages. The question why the TNF-α released under these conditions was not also bound to β-glucan particles and was measurable in the medium arises. During experiments using low and intermediate concentrations of β-glucan (≤200 μg/ml), virtually all particles were phagocytized by the macrophages. However, in experiments employing higher concentrations of β-glucan (500 to 1,000 μg/ml), substantial numbers of free particles were not taken up by the macrophages and remained extracellular after overnight incubation. These nonphagocytized β-glucan particles were available to bind TNF-α released under these conditions, resulting in reduced amounts of TNF-α in the medium.

There exist other potential mechanisms by which high concentrations of fungal β-glucans may suppress release of TNF-α. For example, particulate fungal β-glucans are known to stimulate production of prostaglandin E_2 (PGE_2), an arachidonic acid metabolite which further inhibits TNF-α release (25–27, 44). However, recent studies in our laboratory have not confirmed a role for PGE_2 in modulation of TNF-α release from β-glucan-challenged macrophages. Attempts to enhance PGE_2 activity (dazmegrel treatment) or diminish PGE_2 production (indomethacin treatment) in macrophages exposed to β-glucan did not significantly alter the amount of TNF-α released (unpublished observations). Similarly, Kunkel and coworkers also failed to demonstrate augmented TNF-α release following indomethacin treatment of peritoneal macrophages challenged with zymosan, a fungal derivative composed largely of β-glucan (27). It is therefore unlikely that the suppression of TNF-α induced by high concentrations of β-glucans occurs through alterations in eicosanoid metabolites.

Although TNF-α binds to β-glucan present in fungal cell walls, this interaction does not necessarily result in any direct deleterious effects to the microbe. In recent experiments, we observed that TNF-α (up to 100 ng/ml) did not impair the viability of *S. cerevisiae* organisms (unpublished observations). In contrast, other investigations indicate that TNF-α may exert a direct toxic effect on *P. carinii* and a trypanolytic effect on salvarian trypanosomes (32, 42, 43). TNF-α has also been shown to bind to gram-negative bacteria, including *Shigella flexneri* (33). Under the conditions in that study, the binding of TNF-α to *S. flexneri* is not directly toxic to these bacteria but instead promotes organism invasion of epithelial cells and uptake by macrophages (33). Thus, the biological significance of TNF-α's interaction with a particular microbe is dependent

upon both the organism and the surrounding host cellular milieu.

Our study further evaluated additional mechanisms by which host cells respond to fungal β-glucan. We additionally demonstrated that vitronectin augments the macrophage cytokine response to β-glucan. Vitronectin has been shown to bind to the cell walls of pathogenic fungi, including *P. carinii* and *C. albicans* (30, 31). The interaction of vitronectin with *C. albicans* promotes attachment of the organisms to macrophages (30). Elevated levels of vitronectin have been found in the lungs during *P. carinii* pneumonia, and vitronectin enhances macrophage TNF-α release in response to this organism (36). Our current study further reveals that vitronectin interacts with β-glucans and potentiates macrophage TNF-α release in response to this fungal component. The mechanisms by which vitronectin augments macrophage responses to β-glucan have not yet been established. Vitronectin may agglutinate β-glucan-containing particles and stabilize their adherence to macrophages. It is also conceivable that the coordinate binding of vitronectin to macrophage integrin receptors may heighten the state of intracellular activation, thereby priming the phagocyte for TNF-α release triggered by β-glucans. Furthermore, it remains possible that vitronectin and TNF-α might compete for the same binding sites on fungal β-glucans. Thus, increasing concentrations of vitronectin may displace TNF-α from β-glucan, restoring cytokine activity and rendering it more available for measurement.

In summary, we have demonstrated that β-glucan, a principal component of fungal cell walls, can both stimulate and suppress the release of TNF-α activity from macrophages. Fungal β-glucans bind released TNF-α through lectin-mediated interactions. We additionally demonstrate that the host adhesive glycoprotein vitronectin interacts with β-glucans, thereby augmenting macrophage release of TNF-α. The interaction of fungal β-glucans with vitronectin and macrophages likely represents a significant mechanism of host recognition of fungal pathogens.

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