Linkage Specificity and Role of Properdin in Activation of the Alternative Complement Pathway by Fungal Glycans

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ABSTRACT Fungal cell walls are predominantly composed of glucans, mannans, and chitin. Recognition of these glycans by the innate immune system is a critical component of host defenses against the mycoses. Complement, an important arm of innate immunity, plays a significant role in fungal pathogenesis, especially the alternative pathway (AP). Here we determine that the fungal cell wall glycans chitin and chitosan fail to activate the AP. This study elucidates how the specificity of cell wall glycan linkages affects AP activation and the role properdin plays in this process. Particulate glucans activated the AP even in the absence of properdin. Failure to activate the AP. This study elucidates how the specificity of cell wall glycan linkages affects AP activation and the role properdin plays in this process. Particulate glucans activated the AP even in the absence of properdin, while glucans generated active C3. Properdin colocalized with bound C3, suggesting that in the presence of serum, properdin bound directly to glycans through C3 convertases. These findings provide a better understanding of how properdin facilitates AP activation by fungi through interaction with the cell wall components.

IMPORTANCE Invasive fungal infections have increased in incidence with the widespread use of immunosuppressive therapy and invasive procedures. Activation of the complement system contributes to innate immunity against fungi by generating chemotactic factors that recruit white blood cells and by coating the pathogen with complement fragments that “mark” them for phagocytosis. The fungal cell wall activates complement in an antibody-independent manner through the alternative pathway (AP). Properdin is a positive regulator of the AP. This study elucidates how the specificity of cell wall glycan linkages affects AP activation and the role properdin plays in this process. Particulate glucans activated the AP even in the absence of properdin, while glucans required properdin for AP activation. In contrast, the glucans chitin and chitosan failed to activate the AP. These findings enhance our mechanistic understanding of how fungi activate complement and have implications for the use of glycans in biomedical applications.

Complement activation, a critical component of host defenses against fungal infection (1–3), can proceed by three well-defined pathways. Classical pathway activation is mainly initiated when antibody is complexed to antigens. The lectin pathway shares structural similarities to the classical pathway but is triggered by binding to mannose and ficolins. The third complement activation pathway, the alternative pathway (AP), does not require antibody or lectins for its initiation. Rather, initiation of the AP occurs by spontaneous low-rate hydrolysis of the thioester in C3, which results in continuous supply of C3(H2O) in solution (4). The half-life of the activated C3 thioester has been estimated to be ~60 µs (5). Binding of factor B to C3(H2O) and cleavage of bound factor B to Bb by the enzyme factor D lead to the formation of the initial AP C3 convertase [C3(H2O)Bb]. This fluid-phase C3 convertase can cleave C3 to release C3a and C3b, and the latter can covalently bind to microbial surfaces. Binding of factor B to surface-bound C3b in the presence of factor D leads to the formation of an AP C3 convertase (C3b,Bb) that cleaves additional C3 molecules. This positive C3 amplification loop is a key feature of the AP. The AP C3 convertase C3b,Bb has a half-life of only 1.5 min. Properdin carries out the important function of binding to and stabilizing the C3b,Bb complex, thereby increasing its half-life 5- to 10-fold (6). Properdin is the only known positive regulator of the complement system.

Properdin is found in serum at a concentration of 4 to 25 µg/ml (7–11). Properdin is stored in neutrophils as secondary regulatory proteins in the form of pre-properdin (12). Properdin interacts with membrane-bound C3b,Bb in the presence of serum to form the C3b,Bb, properdin complex (C3b,BbC). This complex, called the AP C3 convertase, cleaves additional C3 and generates C3a and C3b (Figure 1). C3a is an anaphylatoxin that generates inflammatory responses. C3b covalently binds to microbial surfaces, allowing for complement deposition and phagocytosis.

In addition to its role as a positive regulator of the AP, properdin binds to AP C3 convertase (C3b,Bb). Properdin colocalizes with bound C3, suggesting that in the presence of serum, properdin binds directly to glycans through C3 convertases. These findings provide a better understanding of how properdin facilitates AP activation by fungi through interaction with the cell wall components.

These findings enhance our mechanistic understanding of how fungi activate complement and have implications for the use of glycans in biomedical applications.
25 years ago, Czop and Austen (25) demonstrated that aggregated effective design of fungal glycan-based biotherapies. More than host defenses and fungal pathogenicity but also for the safe and activate complement is important not only for understanding particles as a vaccine delivery platform and immunoadjuvant. preparations derived from fungi such as Saccharomyces cerevisiae. Glucan particles (GP), highly purified polysaccharide composition provides receptor-targeted delivery have been suggested as an antigen delivery vehicle because their most fungal cell walls feature an inner layer composed of mannoproteins (16–18). Mannans consist of polymers of mannosyl added to proteins via N- or O-linkages. The β–glucan layer predominantly comprises β-1,3 linkages, with a minority (9 to 20%) of glucans linked by a β-1,6 bond (18, 19). Glucan particles (GP), highly purified Saccharomyces cerevisiae cell walls composed of β-1,6 branched β-1,3 glucans have been suggested as an antigen delivery vehicle because their polysaccharide composition provides receptor-targeted delivery to phagocytic antigen-presenting cells and their hollow and porous structure allows for high antigen loading (20, 21). β-Glucan preparations derived from fungi such as Saccharomyces cerevisiae have a record of safety in both preclinical and human trials (22–24). These findings support the clinical development of glucan particles as a vaccine delivery platform and immunoadjuvant.

Deciphering the mechanistic basis for how fungal cell walls activate complement is important not only for understanding host defenses and fungal pathogenicity but also for the safe and effective design of fungal glycan-based biotherapies. More than 25 years ago, Czop and Austen (25) demonstrated that aggregated β-glucans derived from yeast were potent activators of the human AP. In this study, we have extended those observations and have defined the role of properdin in activating the AP in a linkage-specific manner. These studies provide important mechanistic insights into the role of properdin in complement activation on fungal glucans, which could affect downstream events in the immune response, such as leukocyte recruitment, neutrophil activation, and T cell skewing.

**RESULTS**

C3 is deposited on GP through the AP. Complement-dependent C3 fragment deposition on glucan particles (GP) (contains both β1→3 and β1→6 β-glycosidic linkages) was measured by flow cytometry. The alternative pathway (AP) (normal human serum [NHS] treated with 10 mM EGTA and 10 mM Mg²⁺ [Mg-EGTA-NHS]) accounted for C3 deposition at 30 min; addition of the lectin and classical pathways (NHS) did not increase C3 deposition (Fig. 1, left panel). These results were similar to those observed with zymosan, which is known to activate C3 through the AP (26) and used here as a positive control. Although zymosan displayed the same results qualitatively, less C3 deposition was observed (Fig. 1, right panel). To ensure that chelating Ca²⁺ with EGTA did not affect the assays, we also used C2-depleted serum (AP alone, functional) and confirmed the results (data not shown); Mg-EGTA-treated serum was employed for the remainder of the study.

Properdin facilitates AP-mediated C3 deposition on GP. Properdin is the only known positive regulatory protein of the AP (6), and we speculated that it facilitated deposition of C3 fragments on GP. We used confocal microscopy to assess whether C3 and properdin localized as predicted by the traditional model of properdin binding to alternative pathway C3/C5 convertases (26–28). As a prelude to confocal microscopy experiments, we examined several concentrations of Mg-EGTA-NHS with functioning properdin or Mg-EGTA-NHS where properdin function was blocked with monoclonal antibody (MAb) A233 (Quidel), and we found that a serum concentration of 40% (vol/vol) (40% NHS), which had been optimized for measuring C3 deposition by fluorescence-activated cell sorting (FACS), was also suitable for confocal experiments. GP or zymosan was incubated with either 40% NHS treated with 10 mM EGTA and 10 mM Mg²⁺ (40% NHS–Mg-EGTA-NHS) or 40% human properdin-depleted serum, and the reactions were stopped at 10, 15, 20, and 30 min by adding EDTA. C3 deposition was detected using fluorescein isothiocyanate (FITC)-conjugated anti-human C3c, and bound properdin was detected using anti-human properdin followed by anti-mouse IgG conjugated to Alexa Fluor 647. At 10 min, discrete foci with colocalization of C3 and properdin were observed on both GP and zymosan particles (Fig. 2A). With time, C3 deposition spread over the entire surfaces of the particles, while properdin binding continued at discrete locations of C3b binding (Fig. 2A and B, merged columns). In the absence of properdin, the amount of C3 fragments deposited on both GP and zymosan particles was greatly reduced (Fig. 2B shows minimal C3 deposition at 30 min), highlighting the importance of properdin in facilitating C3 deposition on these particles.

Properdin binds to GP only in the presence of C3 in the context of human serum. Previous studies have shown that native forms of purified properdin can bind directly to certain AP activator surfaces, including zymosan in non-serum-containing sys-
tems (29). Because it has been suggested that a serum component(s) may interfere with binding of properdin directly to alternative pathway activator surfaces (30), we questioned whether properdin could bind directly to GP and zymosan particles in the context of serum. C3-depleted serum was used to eliminate C3 deposition on GP and zymosan to examine whether properdin would bind directly to GP and zymosan surfaces [or possibly through a serum component(s) distinct from C3]. There was no detectable properdin binding to GP or zymosan by FACS analysis in C3-depleted serum over baseline fluorescence levels seen with properdin-depleted serum (Fig. 3A). Reconstitution of C3-depleted serum with physiological concentrations of purified

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**FIG 2** The kinetics and location of C3 deposition and properdin binding on GP and zymosan particles by confocal microscopy. (A) GP and zymosan particles were incubated with 40% NHS–Mg-EGTA (40% NHS treated with 10 mM EGTA and 10 mM Mg²⁺), and the reaction was stopped by adding 10 mM EDTA at the indicated time points. C3 deposited on particles was detected with sheep polyclonal anti-human C3c conjugated to FITC (green) (C3 - FITC columns), and properdin (P) binding was detected with an antiproperdin MAb (A235; Quidel MAb 235) followed by Alexa Fluor 647-labeled anti-mouse IgG (red) (P – A657 columns). The merged columns are the merged images of C3 and properdin binding. (B) Properdin is required for maximal C3 deposition on GP and zymosan. GP and zymosan were incubated with 40% serum (Mg-EGTA-treated serum depleted of properdin). C3 and P binding were detected as described above for panel A. The phase-contrast images are shown in the leftmost column, and the phase-contrast and C3 images were merged and are shown in the rightmost column. The images were taken using a 63× lens and imaged with a Leica TCS SP2 AOBS laser scanning confocal microscope (Leica, Wetzlar, Germany).
C3 (1 mg/ml) restored properdin binding, measured by flow cytometry (Fig. 3A). These results were confirmed using confocal microscopy (Fig. 3B), where properdin bound to GP and zymosan occurred only when active C3 was present in the serum, not when C3-depleted serum was used. These data provide evidence that properdin in human serum does not bind directly to GP or zymosan but associates with alternative pathway C3/C5 convertases that bound to GP and zymosan.

Glycan linkage specificity and influence of properdin on AP-mediated release of C3a and C5a. GP are primarily composed of $1,3$ and $1,6$ glucan linkages (21, 31), while zymosan comprises $1,3$ and $1,6$ $\beta$-glucans and mannans (32, 33). In addition to $1,3$ and $1,6$ linkages, fungal glucans contain a small fraction of chitin composed of $1,4$ glycosidic linkages (34–36). Both GP and zymosan also contain a small fraction of chitin. We examined whether glycan linkage specificity affected activation of the AP using glycan particles from an array of sources, which are listed in Table 1. In addition, three soluble glycans (dextran, laminarin, and mannan), representing several glycan linkages, were used as negative controls because soluble glycans are poor AP activators (25).

**FIG 3** Properdin in serum binds to GP and zymosan particles only in the presence of active C3. Properdin binding to GP and zymosan was detected following incubation of the particles with C3-depleted serum and C3-depleted serum reconstituted with purified C3. The final concentration of serum in all reaction mixtures was 40%. Normal human serum (NHS) and properdin-depleted (P-depleted) serum were used as positive and negative controls for properdin binding, respectively. (A) Glycan-bound properdin was detected by flow cytometry using an antiproperdin MAb (A235; Quidel) followed by anti-mouse IgG conjugated to Alexa Fluor 647. In all graphs, the $x$ axis shows fluorescence on a log$_{10}$ scale, and the $y$ axis shows the number of events. Numbers adjacent to the histogram represent the median fluorescence intensity of properdin binding. (B) Properdin binding to GP and zymosan by confocal microscopy. Using conditions described above for panel A, the particles were stained for properdin using an antiproperdin MAb and Alexa Fluor 647. The phase-contrast images, properdin staining (red) (middle column), and merged images of the phase-contrast and properdin images are shown in the right-hand column. The images were collected and analyzed as described in the legend to Fig. 2. The results of one representative experiment of two separate and reproducibly repeated experiments are shown.

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**Glycan linkage specificity and influence of properdin on AP-mediated release of C3a and C5a.** GP are primarily composed of $1\rightarrow3$ and $1\rightarrow6$ glucan linkages (21, 31), while zymosan comprises $1\rightarrow3$ and $1\rightarrow6$ $\beta$-glucans and mannans (32, 33). In addition to $1\rightarrow3$ and $1\rightarrow6$ linkages, fungal glucans contain a small fraction of chitin composed of $1\rightarrow4$ glycosidic linkages (34–36). Both GP and zymosan also contain a small fraction of chitin. We examined whether glycan linkage specificity affected activation of the AP using glycan particles from an array of sources, which are listed in Table 1. In addition, three soluble glycans (dextran, laminarin, and mannan), representing several glycan linkages, were used as negative controls because soluble glycans are poor AP activators (25).

Activation of AP generates the C3 convertase C3b,Bb that cleaves the $\alpha$ chain of C3 and releases the N-terminal 9-kDa C3a fragment. Cleavage of the C3a fragment is accompanied by exposure of a labile internal thioester bond in the resulting $\alpha'$ chain of C3b. The C3b molecule must bind to a surface target (in this instance, the glycan particle) through a covalent ester or amide bond. Failure to do so results in hydrolysis of the internal thioester, leaving the C3b unbound in solution. Continued complement activation results in formation of the AP C5 convertase (C3bC3b,Bb) that cleaves C5 and releases the C5a fragment. We used enzyme-linked immunosorbent assay (ELISA) to measure the amounts of C3a and C5a generated by particulate and soluble glycans that activated the AP. Glucan particles that contained only $1\rightarrow3$ linkages (curdlan) or $1\rightarrow6$ linkages (pustulan) or both $1\rightarrow3$ and $1\rightarrow6$ linkages (GP and scleroglucan) were potent activators of AP, generating high levels of C3a and C5a (Fig. 4A and C). In contrast, particles that comprised only $1\rightarrow4$ linkages of amino $N$-acyetylglucosamine and $N$-glucosamine (chitin and chitosan) failed to significantly activate AP above baseline levels. As expected, zymosan, a known AP activator that contains both $\beta$-glucans and mannans, generated high levels of C3a and C5a.
The impact of C3 and properdin binding on GP (high C3, low properdin [Fig. 1 and 3]) and zymosan (low C3, high properdin [Fig. 1 and 3]) was reflected in the generation of C3a and C5a by the AP with functional properdin. Zymosan generated 78% of C3a and 93% of C5a production achieved by GP only in serum with intact AP that contained active properdin (Fig. 4A and C). In accordance with previous work (25), none of the soluble glycans activated AP significantly above activation of AP by serum alone.

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only particles that contained β1→3 linkages (curdlan, GP, and scleroglucan) generated C3a and C5a above baseline levels when properdin function was blocked. Pustulan (β1→6 linkages only) did not activate AP in the absence of properdin function (Fig. 4B). Although the total amounts of C3a and C5a generated in the absence of functional properdin were, on average, 10-fold less than when properdin was active, it is worth noting that the amounts of C3a and C5a spontaneously generated in the control reactions (serum plus buffer alone) when properdin was nonfunctional were ~30-fold and ~5-fold less for C3a and C5a generation, respectively (Fig. 4A and B and Fig. 4C and D, respectively). As expected, chitin, chitosan, and the soluble glycans did not generate C3a and C5a when properdin was nonfunctional. Interestingly, zymosan and GMP (both consisting of β-glucans, including β1→3 and β1→6 glucans and mannans) generated C3a and C5a in the presence of Mg-EGTA-treated NHS (properdin active) but did not significantly do so in the absence of properdin. Similar C3a and C5a generation were observed using Mg-EGTA-treated properdin-depleted serum (data not shown).

**Influence of properdin on the deposition of C3 fragments onto glycan particles.** Deposition of C3b on the surface of a glycan particle reflects the number of proximate –OH groups that can serve as electron donors to form covalent ester bonds with activated C3b. We characterized C3 deposition on different particles by Western blotting. The 75-kDa β chain present in both C3b and iC3b, freed by reducing conditions prior to SDS-PAGE and Western blotting, is a measure of total C3 deposited onto particles (Fig. 5A). The amount of C3 deposited onto a particle may be paralleled by the amount of C3a and C5a generation, respectively (Fig. 4A and B and Fig. 4C and D, respectively). As expected, chitin, chitosan, and the soluble glycans did not generate C3a and C5a when properdin was nonfunctional. Interestingly, zymosan and GMP (both consisting of β-glucans, including β1→3 and β1→6 glucans and mannans) generated C3a and C5a in the presence of Mg-EGTA-treated NHS (properdin active) but did not significantly do so in the absence of properdin. Similar C3a and C5a generation were observed using Mg-EGTA-treated properdin-depleted serum (data not shown).

FIG 5 C3 fragment deposition on the β-glycan particles. (A) Schematic representation of C3 degradation products. (B) Particles were incubated with 40% NHS–Mg/EGTA, washed, and digested with 4× LDS sample buffer containing 10% β-mercaptoethanol. Samples were electrophoresed on a 4 to 12% Bis-Tris gel, and Western blotting was performed. C3 fragments were identified using affinity-purified sheep anti-human C3 antibody. Controls included 2 μl of the 200× diluted NHS-Mg-EGTA (labeled Control), purified C3, C3b, and iC3b (6.25 ng of each protein per lane). (C) C3 fragments bound to the glycan particles in the absence of functional properdin. The particles were incubated in 40% NHS–Mg/EGTA that contained antiproperdin MAb 233 (anti-P mAb) and processed as described above for panel B, and Western blotting was performed as described above. Controls are described above for panel B, except that all specimens contained antiproperdin MAb 233. The identities of the C3 fragments are indicated in parentheses on the right (α C3). The results of one representative experiment of three separately performed and reproducibly repeated experiments are shown. kD, kilodaltons.
or no C3 was deposited when properdin was blocked on pustulan (β1→6 glucan) and on zymosan and GMP (Fig. 5B and C). In the absence of functional properdin, only the β1→3 linkage-containing glucans (curdlan, GP, and scleroglucan) bound C3, consistent with their ability to generate C3a and C5a under these conditions (Fig. 4B and D); minimal C3 was deposited on pustulan, zymosan, and GMP in the absence of functional properdin.

Collectively, our data suggest that when properdin is functional, β1→3 and β1→6 linkage-containing glucans are potent activators of the alternative pathway. However, in the absence of functional properdin, only β1→3 linkage-containing glucans can activate the alternative pathway, albeit to a much lower level than when properdin is functional. In the case of zymosan and GMP, β1→3 glycan chains that are present may be less exposed at the surface because the outer mannan “layer” shields the glycan from interacting with C3 molecules.

**DISCUSSION**

We have defined how monosaccharide composition and linkage specificity of β-glucans (β1→3 and β1→6 linked) together influence activation of the alternative pathway (AP). Importantly, the role played by properdin in linkage-specific β-glucan-mediated AP activation has also been defined. β-Glucans are found naturally in fungi, algae, plants, and some bacteria; both β1→3 and β1→6 glucans are major constituents of the cell walls of fungi. A small percentage of the fungal cell wall is composed of chitin (β1→4 N-acetylglucosamine). As shown previously (25), we also demonstrated that all glucan particles (GP) with either β1→3 and/or β1→6 glucan linkage activated C3 when the AP was intact. In contrast, chitin or chitosan (β1→4 linkages) activated the AP (measured by C3a and C5a generation) only minimally. In the absence of functional properdin, only glucan particles that express surface-exposed β1→3 linkages (GP, scleroglucan, and curdlan) generated C3a and C5a, albeit at concentrations about 10-fold lower than when properdin was functional. Pustulan (contains only β1→6 glucan linkages) was a potent AP activator in the presence of properdin but generated minimal C3a and C5a when properdin function was blocked. These data suggest that glucan particles that express surface-exposed β1→3 glucan linkages can activate C3 in the absence of properdin, but particles with only β1→6 glucan linkages require properdin to activate C3.

Following C3 activation, the metastable C3b molecule must quickly bind to an electron-donating −OH group through the thioester bond; failure to do so results in hydrolysis of the thioester, and the C3b molecule remains in solution. Free C3b can form alternative pathway C3 and C5 convertases (C3bBb and C3bC3bBb) in solution that can further activate C3 and C5 and generate more C3a and C5a, respectively. However, the amount of C3b deposited on the glycans depends on the availability of hydroxyl (−OH) groups on ringed monosaccharides, principally hexoses, and serves to explain the apparent discordance between C3a and C5a generation (Fig. 4A and C) and C3 fragment deposition (Fig. 5B). The particulate β1→3 glucans used in this study exist primarily in a triple helix conformation (37). Based on the results of glucan fiber diffraction studies (37), the hydroxyl groups at positions 2 and 4 are buried within the polysaccharide chain participating in intra- and interchain hydrogen bonds. The hydroxyl at position 6 are exposed to solvent and are the likely electron donors for reaction with C3. Removal of primary −OH groups from position 6 of monosaccharides decreases the efficiency of C3 binding (38). Consistent with our observations (Fig. 5B), the β1→3 particulate glucans with exposed free −OH groups at position 6 (curdlan, GP, and scleroglucan) bound C3 more effectively than β1→6 glucans, whose position 6 is occupied by the linkage to the adjacent monosaccharide (pustulan). Also consistent with our observations, the β1→3 particulate glucans with free −OH groups at position 6 masked by mannan (zymosan and glucan-mannan particles [GMP]) bound C3 less effectively. Higher efficiency of β1→3 glucan binding to nascent C3 likely facilitates C3b binding to −OH at position 6, even when the AP lacks properdin (Fig. 5C).

Release of anaphylatoxins C3a and C5a during activation of complement has also been shown to promote neutrophil influx to sites of infection. Human neutrophils release properdin upon stimulation (12), which likely serves to augment AP activation (39). β1→6 glucans, in particular, stimulate human neutrophils and mediate engulfment, production of reactive oxygen species, and expression of heat shock proteins more efficiently than β1→3 glucans (40). In one study, human neutrophils rapidly ingested beads coated with β1→6 glucan, but not beads coated with β1→3 glucan (40), suggesting that stimulation of neutrophils may be directed by the specific location of C3b binding on glucan surfaces. The effects may not be limited to neutrophils, as recently, it was shown that C3a and C5a generation can regulate interleukin 17A (IL-17A) responses (41), a critical component of host defense against many fungal infections. Selective reduction of properdin function may be of therapeutic benefit. For example, in a model of cardiopulmonary bypass, treatment with antiproperdin antibodies resulted in significantly reduced neutrophil and platelet activation (14). However, our data suggest that neutralizing properdin could increase the risk of fungal infections by decreasing AP activation.

In addition to affecting innate immune responses, generation of C3a and C5a can affect adaptive immune responses (41, 42). For example, recently, Lajoie et al. demonstrated the abilities of C3a and C5a to reciprocally regulate T17 (41) and T17 responses in an airway hyperresponsiveness mouse model (41). As T17 and T17 responses are critical components of host defense against many fungal infections (43, 44), the contribution of complement activation by fungal cell walls to TH17 skewing deserves further study.

We have shown that purified properdin in its native forms (dimers, trimers, or tetramers) binds directly to zymosan (27, 29), but data from the present study suggest that properdin does not bind directly to either GP or zymosan in the context of serum but instead binds to deposited C3 (Fig. 3A and B). Thus, it seems unlikely that properdin itself initiates AP activation in the presence of serum, confirming that properdin serves to stabilize AP C3 convertases (26–28). Serum amyloid P component has been reported to interfere with properdin’s ability to bind to complement activator surfaces (30). Although zymosan and GMP contain both β1→3 and β1→6 glucan linkages and activated the AP in the presence of properdin, C3 was minimally activated by these particles in the absence of properdin. We speculate that mannan present on the surfaces of zymosan and GMP may limit covalent linkage formation between nascent C3b and the underlying β1→3 glucans. The finding that properdin is required for AP activation by mannan-coated particles may be clinically relevant, considering the fact that most pathogenic fungi have an outer mannan layer.
Because hollow and porous β-glucan particles allow for high antigen loading (20, 21, 35), they can serve as an effective antigen-presenting cell receptor-targeted vaccine delivery system. Recently, orally administered β-glucan particles have also been shown to function as effective adjuvants for tumor immunotherapy (45). Although the relative contributions of complement receptors and β-glucan receptors remain to be defined, efficient coating of β1–3 linkage-containing particles such as GP with C3 fragments likely contributes to their excellent ability to act as an adjuvant. Goodridge et al. (46) recently demonstrated that Dectin-1, a pattern recognition receptor, was activated only by particulate β-glucans and not by soluble β-glucans (46). Particulate β-glucans clustered around the receptor to form synapse-like structures activating phagocytosis (46). Binding of C3 fragments to antigens facilitates their uptake by subcapsular sinus macrophages in lymph nodes (47). Transfer of the antigen–C3 fragment complexes to mature B cells involves binding of the C3d-coated antigen to the B cell coreceptor (CD21/CD19/CD81), which lowers the threshold for B cell activation (48) and results in B cell expansion and migration to the T cell-B cell boundary in lymph nodes. Following interaction with follicular T helper cells, B cells undergo somatic cell hypermutation and class switch recombination and develop into plasma cells or memory B cells (reviewed in reference 47). Our observations regarding how properdin facilitates AP activation by glucans in fungal cell walls contributes to our understanding of the complexity of complement activation by fungi and has implications for the use of glucan particles in biomedical applications.

**MATERIALS AND METHODS**

**Particles used in this study.** Glucan particles (GP) and glucan-mannan particles (GMP) were prepared from Saccharomyces cerevisiae using a succession of alkaline and acidic extraction steps as previously described (21, 31, 35, 36). GP are comprised of >85% β1–6 branched β1–3 β-glucan polymers, 2% chitin, and <1% lipids and protein, with the remainder being mostly ash and moisture, and GMP are comprised of ~50% β1–6 branched β1–3 β-glucan polymers, ~20% mannan, ~15% protein, 1% chitin, and <1% lipids (31, 49). Zymosan (β-glucans, mannan, and chitin), also prepared from S. cerevisiae, was obtained from Sigma (St. Louis, MO). All particulate and soluble glucans used in this study and their source and linkage composition are listed in Table 1.

**Sera and complement reagents.** Hemolytically active sera obtained from 10 healthy adults (normal human serum [NHS]) were pooled and stored at ~80°C until used. To permit selective activation of the alternative pathway (AP), NHS was treated with 10 mM EGTA and 10 mM Mg²⁺ (Mg-EGTA-NHS). The role of properdin in AP was determined by using Mg-EGTA-treated properdin-depleted serum (P-depleted serum; catalog no. A512; Quidel), or Mg-EGTA-NHS containing anti-properdin monochlonal antibody (MAb) (catalog no. A233 [see below]; Quidel) used at a final concentration of 50 µg/ml as described previously (27). Serum depleted of C3 by immunoaffinity chromatography was purchased from Complement Technology, Inc., Tyler, TX (catalog no. A314). C3 was purified from human plasma by polyethylene glycol (PEG) precipitation and DEAE Sephacel chromatography as described previously (50). The final concentration of serum in all reaction mixtures was 40%. In some experiments, all complement pathways were inactivated by heating serum for 30 min at 65°C.

**Antibodies.** Two antiproperdin MAb s were purchased from Quidel (catalog no. A233 and A235). MAB A235 blocks properdin function, while MAB A233 binds to properdin but does not block the function of properdin. C3 (C3b plus iC3b) deposition on GP and zymosan was detected by anti-human C3c conjugated to fluorescein isothiocyanate (FITC) from BioDesign (now Meridian Life Science, Inc., Saco, ME) for both flow cytometry and confocal microscopy. For Western blot analysis, C3 deposition was detected using affinity-purified sheep anti-human C3 antibody (Immunology Consultants Laboratory, Newburg, OR) followed by anti-sheep IgG alkaline phosphatase (Sigma).

**C3 deposition and properdin binding to GP and zymosan by flow cytometry.** C3 deposition on GP and zymosan was determined by flow cytometry. Briefly, 10 µg of GP or zymosan particles were suspended in Hanks’ balanced salt solution (HBSS). Because GP and zymosan particles are similarly sized and shaped, an approximately equal amount of surface area should have been available for both types for C3 deposition. The particles were washed once with HBSS containing 10 mM EGTA and 10 mM Mg²⁺ (HBSS-Mg-EGTA) and incubated for 30 min at 37°C with either NHS (all complement pathways active), Mg-EGTA-NHS (only AP active), or heat-inactivated NHS (complement activity inactivated). A reaction mixture that lacked serum was also included as a control. Total C3 (C3b plus iC3b) deposited on GP or zymosan was detected by flow cytometry using FITC-conjugated anti-C3c antibody as described previously (51, 52). To demonstrate the dependence of C3 deposition on GP or zymosan for properdin binding, particles were incubated with C3-depleted serum (Complement Technologies, Inc.) or C3-depleted serum reconstituted with C3 to a concentration of 1 mg/ml, and binding of properdin was determined using anti-human properdin MAB (catalog no. A235; Quidel) at a dilution of 1:100, followed by anti-mouse IgG conjugated to Alexa Fluor 647 (1:400) as described previously (27). Positive and negative controls included particles incubated with Mg-EGTA-NHS or Mg-EGTA-treated, properdin-depleted serum (Quidel), respectively. Data were collected on an LSR II flow cytometer (Becton Dickinson [Franklin Lakes, NJ]) and analyzed using the FlowJo analysis software program (version 7.2.4; TreeStar Inc. [Ashland, OR]).

**Confocal microscopy.** C3 fragment deposition on and properdin binding to GP and zymosan were also examined by confocal microscopy. Briefly, 10 µg of GP or zymosan was washed once with HBSS-Mg-EGTA and incubated with either Mg-EGTA-NHS or Mg-EGTA-treated, properdin-depleted serum for time points between 10 and 30 min. The particles were washed three times, and C3 and properdin associated with GP or zymosan were detected using FITC-conjugated anti-C3c and anti-human properdin MAB (catalog no. A235; Quidel) at a dilution of 1:100, followed by anti-mouse IgG conjugated to Alexa Fluor 647 (1:400). The stained particles were resuspended in HBSS containing 1% paraformaldehyde and spotted onto a 35-mm petri dish with a 10-mm microwell (MatTek Corporation, Ashland, MA) and imaged using a 63X plan apo-achromat objective lens (Zeiss) with an inverted TCS SP2 AOBS laser scanning confocal microscope (Leica, Wetzlar, Germany). To demonstrate the dependence of C3 deposition on GP or zymosan for properdin binding by confocal microscopy, the particles were incubated with C3-depleted serum that had been treated with Mg-EGTA (C3-depleted serum-Mg-EGTA) or C3-depleted serum-Mg-EGTA reconstituted with purified C3 (1 mg/ml) at 37°C for 30 min. Positive and negative controls included incubation of particles with Mg-EGTA-NHS and Mg-EGTA-treated, properdin-depleted serum, respectively. The particles were washed three times, and properdin associated with GP or zymosan was detected using anti-human properdin MAB (catalog no. A235; Quidel) at a dilution of 1:100, followed by anti-mouse IgG conjugated to Alexa Fluor 647 (1:400). The stained particles were resuspended in HBSS containing 1% paraformaldehyde and imaged as mentioned above.

**C3a and C5a assays.** Activation of C3 is accompanied by release into solution of the C3a fragment from the N terminus of the α chain of C3. Furthermore, AP activation results in formation of C5 convertase (C3bC3b,Bb) that cleaves C5 and releases the C5a fragment. Briefly, 10 µg of each glycanchain of glycans or soluble glycans (listed in Table 1) was incubated at 37°C for 30 min with 40% NHS–Mg-EGTA alone or with 40% NHS–Mg-EGTA containing MAB 233, which blocks properdin function, or 40% NHS–Mg-EGTA depleted of properdin. Reactions were stopped by adding EDTA to a final concentration of 20 mM, and particles were separated from the supernatants by centrifugation at 10,000 × g for 5 min.
The amounts of C3a and C5a present in the supernatants were measured using the MicroVue C3a enzyme immunoassay (EIA) kit (catalog no. A015; Quidel) or the MicroVue C5a EIA kit (catalog no. A021; Quidel), respectively.

**AP-mediated C3 deposition on glicyan particles.** C3 fragment deposition on glicyan particles was determined by Western blotting as described previously (53). Briefly, 10 μg of each of the particles listed in Table 1 was incubated with either Mg-EGTA-NHS- or Mg-EGTA-treated serum containing antiprotein C MAb 233 (final serum concentration of 40% [vol/vol]) in a final reaction volume of 100 μl for 30 min at 37°C. The particles were washed four times in HBSS and lysed in 4X lysis buffer (LDS) sample buffer (Invitrogen) containing 10% 2-mercaptoethanol (2-ME). The proteins were separated on NuPAGE Novex 4 to 12% Bis-Tris gradient gels using NuPAGE 3-morpholinopropanesulfonic acid running buffer (Invitrogen). The proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA), and C3 fragments were detected using affinity-purified sheep anti-human C3 antibody followed by goat anti-sheep IgG conjugated to alkaline phosphatase.

**Statistical analysis.** Differences between C3a and C5a generation were calculated between the C3a and C5a generated by NHS-Mg-EGTA in the phatase.

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