β-Glucan Is a Fungal Determinant for Adhesion-Dependent Human Neutrophil Functions[1]

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***Candida albicans* is a common cause of nosocomial infections whose virulence depends on the reversible switch from blastoconidia to hyphal forms. Neutrophils (or polymorphonuclear leukocytes (PMNs)) readily clear blastoconidia by phagocytosis, but filaments are too long to be ingested. Mechanisms regulating immune recognition and response to filamentous fungal pathogens are not well understood, although known risk factors for developing life-threatening infections are neutropenia or defects in the NADPH oxidase system. We show human PMNs generate a respiratory burst response to unopsonized hyphae. Ab specific for β-glucan, a major component of yeast cell walls, blocks this response, establishing β-glucan as a key molecular pattern recognized by PMNs in response to *C. albicans*. This study also elucidates recognition and signaling mechanisms used by PMNs in response to β-glucan under conditions where phagocytosis cannot occur. Human PMNs adhered to immobilized β-glucan and released an efficient plasma membrane respiratory burst. Ab blockade of the integrin complement receptor 3 (CD11b/CD18) significantly inhibited both of these functions. Furthermore, we show a role for p38 MAPK and actin but not protein kinase C ζ in generating the respiratory burst to β-glucan. Taken together, results show that β-glucan in *C. albicans* hyphae is accessible to PMNs and sufficient to support an innate immune response. *The Journal of Immunology,* 2006, 177: 8667–8675.**

Candida species are fungal pathogens reported to be the fourth most common cause of nosocomial bloodstream infections (1). The incidence of infections due to *Candida* is accompanied by a high mortality rate, despite the development of new therapeutics (2). A key virulence factor in the pathogenesis of fungal infections is the reversible morphologic transition of yeast forms, also known as blastoconidia, to filamentous or hyphal forms (3). Immune effector mechanisms underlying the host response to the pathogenic hyphal form of fungi are not nearly as well understood as the response to the yeast form that is subject to phagocytic clearance and intracellular killing.

β-Glucans are a class of long-chain polymers of glucose in β-(1,3) (1,6) linkages found in the cell wall of fungi. Glucans are conserved microbial structures not found in mammalian cells, and are thus considered a pathogen-associated molecular pattern (PAMP)[3] (4). Accumulating experimental evidence points to β-glucan as a key recognition element for mediating macrophage phagocytosis of zymosan, *Pneumocystis carinii*, and *C. albicans* in yeast form, but not in filamentous form (5–8). Among the components of host defense, polymorphonuclear leukocytes (PMNs or neutrophils) are cells known to respond to unopsonized hyphae with a respiratory burst (9) and are so essential to the primary cellular response against fungal infections that neutropenia and PMN dysfunction are primary risk factors in development of *Candida* infection (10, 11). Whether human PMNs perceive the cell wall β-glucan as a PAMP on the invasive, filamentous form of *C. albicans* has not been determined.

Several distinct mammalian phagocytic receptors for β-glucan have been identified, including complement receptor 3 (CR3) (12, 13), lactosylceramide (14), scavenger receptors (15), murine dectin-1 (16, 17), and its human equivalent β-glucan receptor (βGR) (4). The adhesion receptor CR3 (CD11b/CD18) is a member of the β_2 family of integrins and consists of an α-chain (CD11b) noncovalently associated with a β subunit (CD18) expressed on the plasma membranes of mammalian phagocytes and NK cells (reviewed in Ref. 18). On granulocytes, CR3 is important for the recognition of iC3b-opsonized microbial pathogens, with a prominent role in phagocytosis, degranulation, and respiratory burst (19–21). CR3 is structurally unique among integrins in that the α-subunit not only contains a binding site for ligands such as fibrinogen, ICAM-1 and iC3b, but also a spatially separate binding domain for carbohydrates, which serves as a receptor for β-glucan (13, 19, 22). Interactions with the lectin-like site initiate transmembrane signaling that ultimately mediates cytoskeleton-dependent functions such as phagocytosis, chemotaxis, and diapedesis (23–25). In this report, we tested the hypothesis that β-glucan is sufficient to stimulate PMN immune function under conditions in which phagocytosis cannot occur.

One hallmark of the innate immune response to infection is rapid activation of PMNs to produce reactive oxygen intermediates such as superoxide anion (O_2^-) (26). This reactive oxygen intermediates-producing respiratory burst is mediated by the NADPH oxidase complex and results from a number of highly coordinated signaling pathways (27, 28). Phosphorylation of the NADPH oxidase complex in response to soluble stimuli such as fMLP is regulated in part by p38 MAPK (29). The MAPK phosphorylates a number of substrates, including cytoskeletal proteins (30, 31) and transcription factors (32) involved in inflammatory processes. In

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[3] Abbreviations used in this paper: PAMP, pathogen-associated molecular pattern; CR3, complement receptor 3 (CD11b/CD18, Mac-1, or $\alpha_M\beta_2$ integrin); dPBS, Dulbecco's PBS; O_2^-, superoxide anion; Pam_3CSK_4, $Pam_3CysSerLys_4$; PKCζ, protein kinase C ζ; PMN, polymorphonuclear leukocyte or neutrophil; ROS, reactive oxygen species; sBLP, synthetic bacterial lipopeptide; WGP, whole glucan particle; βGR, β-glucan receptor.

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addition, the ζ isoform of protein kinase C (PKC) is also reported to have a role in signaling fMLP activation of NADPH oxidase by phosphorylating specific serine residues on the cytosolic $p47^{phox}$ protein (33). It is not known whether p38 and/or PKCζ mediate the respiratory burst to β-glucan.

In this report we demonstrate that, in the absence of phagocytosis, β-glucan serves as a PAMP in fungal hyphae, which is capable of efficient stimulation of an adhesion-dependent respiratory burst by human PMNs. This activity includes recognition of β-glucan by CR3 and signaling through p38 MAPK, but not PKCζ.

Materials and Methods

Reagents

Highly purified (34) endotoxin-free, soluble yeast β-glucan (PGG-glucan), and whole glucan particles (WGPs) isolated from *Saccharomyces cerevisiae* were provided by Biothera (originally Alpha-β Technology). The PGG-glucan preparation contained <0.02% protein, <0.01% mannan, and 1% glucosamine (35). Mannan (also from *S. cerevisiae*), laminarin, chitin, zymosan, dextran (80–120 kDa), low-endotoxin BSA, PMA, Histopaque 1077 (Ficoll-Hypaque), fMLP, polymyxin B sulfate, ferricytochrome C, cytochalasin B, and aprotinin were purchased from Sigma-Aldrich. Human fibronectin was from BD Biosciences. Pustulan, SB203580, and diisopropyl fluorophosphate were from Calbiochem. Dulbecco's PBS (dPBS), HBSS, Liebovitz's L-15 medium, and DNase were obtained from Invitrogen Life Technologies. $Pam_3CysSerLys_4$ (Pam_3CSK_4 or synthetic bacterial lipopeptide (sBLP)) was purchased from EMC Microcollections and sonicated for 5 min in a water bath before each use. The fluorochromes CFSE and 5-(and-6) chloromethyl-2′,7′-dichlorodihydrofluoroscein diacetate acetyl ester (CM-H_2DCFDA) were purchased from Molecular Probes. PKCζ was inhibited with a myristoylated peptide pseudosubstrate purchased from BioSource International. Abs used were: LM2/1 (Bender Medical Systems); VIM12 (Caltag Laboratories); IB4 (Ancell); ICRF44 (BioSource International); MEM48, VLA-4, and VLA-5 (Chemicon). Hybridoma-derived RP3, specific for rat PMNs, was a gift from Sekiya and colleagues (Yamagata University School of Medicine, Yamagata, Japan) (36). BFDiv, a mouse IgM mAb specific for β-(1-3)- and β-(1-6)-linked glucose, was provided by Biothera (~8.8 mg/ml). The Ab generation and specificity is described in Milton et al. (37) and confirmed in this report. Additional reagents for immunostaining were: saponin (Eastman Organic Chemicals); murine IgM (clone TEPC183; Sigma-Aldrich); FITC-conjugated $F(ab')_2$ anti-mouse IgM (Sigma-Aldrich) and Texas red-conjugated sheep anti-rabbit IgG (BioSource International). All reagents used contained <0.1 pg/ml endotoxin as determined by *Limulus* amebocyte lysate screening (BioWhittaker).

Yeast preparation

C. albicans was obtained from American Type Culture Collection (Wasson, strain 24433) and streaked onto Sabouraud agar plates (Difco) and allowed to form colonies overnight at 37°C. A single colony was picked, seeded into 10 ml of yeast extract-peptone-dextrose medium consisting of yeast extract, bacto-peptone (both from Difco), and dextrose (Sigma-Aldrich) and grown overnight at 37°C with vigorous agitation (225 rpm) on a platform shaker. After culture in yeast extract-peptone-dextrose, blastoconidia were washed with PBS and counted manually on a hemacytometer under ×10 magnification, then induced to form filaments via inoculation at 5×10^5 cells/ml in Medium 199 supplemented with Earle's balanced salt solution, L-glutamine, and 25 mM HEPES (BioWhittaker) and incubation at 37°C for 4–6 h. The filamentous phenotype was confirmed by light microscopy.

Systemic C. albicans *infections*

Male Fisher-344 rats (150–200 g; viral Ag-free plus; Charles River Laboratories) were housed in barrier cages and fed rat chow and water ad libitum. Viral Ag-free plus rats were certified free of common rat pathogens by the supplier and were monitored by Brown University/Rhode Island Hospital veterinary personnel. All animals were handled in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Rats were infected with *C. albicans* by tail vein injection of 3×10^6 CFU in 100 μl of saline. Animals were euthanized 6 days later, and kidneys were fixed in 3% neutral-buffered formalin before dehydration and paraffin embedding.

Immunofluorescent staining of tissues

Immunofluorescent staining of *Candida*-infected kidneys was performed on 6 μM formalin-fixed, paraffin-embedded tissue sections. Sections were deparaffinized in xylene and rehydrated with ethanol and PBS, then were blocked and permeabilized in PBS containing 10 mg/ml BSA and 0.2% saponin for 30 min at room temperature. BFDiv Ab was used (1/100) to recognize β-glucan within the cell wall of *Candida* hyphae, and murine IgM was used as an isotype specificity control. Tissue neutrophils were identified using mouse anti-rat neutrophil RP3 (IgG isotype). Slides were washed and incubated for 30 min at room temperature with FITC-conjugated goat $F(ab')_2$ anti-mouse IgM (1/100) and Texas Red-conjugated sheep anti-rabbit IgG (1/500). Sections were observed using a Nikon Microphot-FXA and images captured using a SenSys charge-coupled device fitted with PVCAM acquisition software (Photometrics).

Immunofluorescent staining of β-glucan on viable C. albicans

C. albicans blastoconidia and hyphae were prepared as above then gently transferred to microtubes then rinsed briefly with PBS and blocked with 3% BSA in PBS for 30 min. The blocking solution was replaced with 3% BSA containing BFDiv or murine IgM for another 30 min at room temperature. Samples were then washed in PBS and incubated for 30 min at room temperature with FITC-conjugated goat $F(ab')_2$ anti-mouse IgM (1/100). Image acquisition as above.

Cell preparation

Neutrophils were isolated from healthy human volunteers by collection into EDTA-containing Vacutainer tubes (BD Biosciences). Histopaque was used for initial cell separation followed by gravity sedimentation through 3% dextran. Contaminating erythrocytes were removed by hypotonic lysis, yielding a neutrophil purity of >98%. PMN were suspended in HBSS for adhesion assays or indicator-free HBSS for oxidative burst assays.

Immobilization of polysaccharides

Varying concentrations of β-glucan or dextran were diluted into dPBS and plated in volumes of 100 μl onto a Costar 96-well Universal Binding polystyrene plate, manufactured with a photoactivatable cross-linking agent (Corning). The plate was incubated at 37°C in a humidified incubator with 5% (v/v) CO_2 for 60 min. After incubation, the plate was flicked to remove liquid and briefly irradiated in a UV Stratalinker 1800 (Stratagene) to activate the surface of the plates, facilitating the formation of a covalent linkage with passively adsorbed carbohydrates. The method has been shown to have an approximate binding efficiency of 10–15% as described previously (35) and confirmed here by phenol-sulfuric acid assay (data not shown). For consistency, the applied concentration is referred to throughout this report. After cross-linking, plates were blocked with endotoxin-free BSA for 20–30 min in a 37°C incubator with 5% (v/v) CO_2, at a concentration of 10 μg/ml for burst assays or 1 mg/ml for adhesion assays. Following incubation, wells were washed twice with dPBS.

Oxidative burst assays

Respiratory burst activity was determined by measuring O_2^- production through its reduction of ferricytochrome C. Neutrophils were plated at a concentration of 3×10^5 cells/well, in replicates of 3–6 wells, at a volume of 100 μl/well onto Costar Universal Binding plates containing immobilized β-glucan or dextran. WGP and zymosan were sonicated to produce a single particle suspension and PMA was used at 200 nM as a positive control. These stimulants were used in the absence of immobilized polysaccharides in wells blocked with endotoxin-free BSA. When indicated, cells were also stimulated with either 10^{-6} or 10^{-7} M fMLP or 5 μg/ml Pam_3CSK_4, a sBLP. To block CR3, cells were incubated with 5 μg/ml LM2/1, MEM48, or VIM12 mAbs for 30 min on ice before being plated. In PKCζ and p38 MAPK inhibition studies, cells were treated with a range of inhibitor concentrations for 10 min at room temperature before adding to ferricytochrome C that contained the identical inhibitor concentration. In studies using cytochalasin B, a final concentration of 2 μg/ml of the reagent was added directly to assays without preincubation. Control wells included superoxide dismutase (300 U/ml), which inhibited reduction of ferricytochrome C by O_2^-. Finally, 100 μl/well 100 μM ferricytochrome C was added to each well and absorbance was measured every 10 min for 60–90 min at dual wavelengths of 550 and 630 nm in a heated Microplate BIO Kinetics Reader (Bio-Tek Instruments), using DeltaSoft3 software. Superoxide production was calculated for 60 min (unless otherwise indicated) from the change in absorbance and the extinction coefficient of NADP(H).

To measure the generation of reactive oxygen species (ROS) by PMN against live yeast, PMN were suspended in HBSS (5×10^6/ml) containing 8 μM CM-H_2DCFDA and equilibrated at room temperature, in the dark for

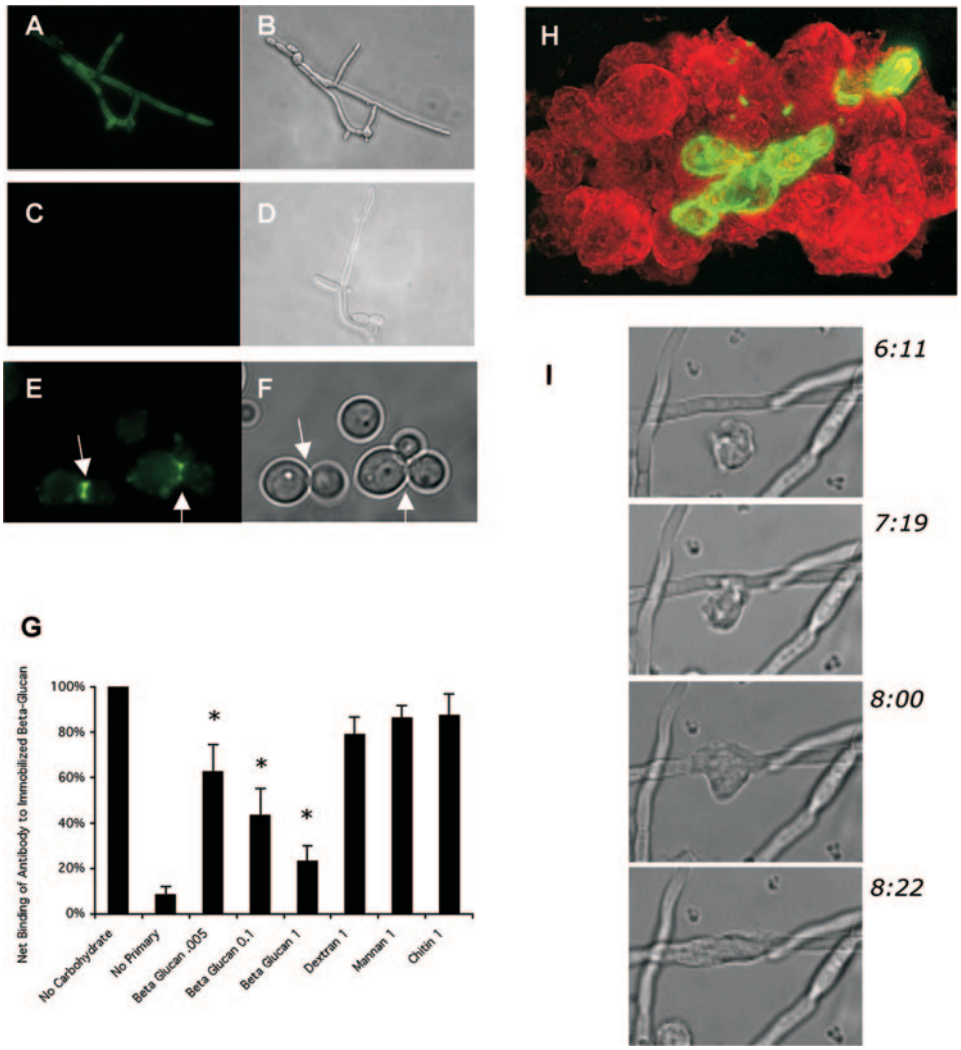

FIGURE 1. In vivo and ex vivo evidence for proximity of PMNs to β-glucan within *C. albicans* cell walls. *A*, Indirect immunofluorescence of yeast grown in culture stained with β-glucan-specific Ab (BFDiv). *B*, Bright field image of same organism. *C*, Yeast stained with IgM as Ab specificity control. *D*, Bright field image of same organism. *E*, Blastoconidia stained with BFDiv. *F*, Bright field image of same organism. *G*, ELISA demonstrating BFDiv specificity determined by ability of BFDiv preadsorbed with soluble β-glucan and other polysaccharides (in mg/ml) to bind immobilized β-glucan. Values are averages $\pm$ SD of two to four experiments with 8-well replicates; $*$, $p < 0.01$. *H*, Indirect immunofluorescence of rat kidney infected with *C. albicans*: yeast was stained with BFDiv and FITC-conjugated anti-IgM; surrounding cells were stained with neutrophil-specific RP3 and Texas red-conjugated anti-IgG. *I*, Sequential real-time images of PMN adhering to unopsonized *C. albicans* hyphal filament; time indicates minutes:seconds. All images taken with an original magnification of $\times$40.

30 min. The compound is a cell-permeant indicator that becomes fluorescent upon oxidation. Cells were washed once in an excess volume of HBSS without cations and suspended to a concentration of 3×10^6/ml in HBSS. *C. albicans* hyphae were grown on 96-well plates coated with fibronectin to promote adhesion to the plate surface and minimize disturbance through multiple assay steps. Plates were prepared by incubating with 100 μl of 10 μg/ml fibronectin diluted in water for 1 h at 37°C, flicking to remove solution, and washing once with PBS. After preparation as described above and enumerating on a hemacytometer, 7×10^3 CFU of *C. albicans* in 100 μl of Medium 199 were added to each well and allowed to grow overnight at 37°C. One hour before use in burst assay, yeast supernatant was removed by aspiration and replaced with 100 μl of blocking solution consisting of 100 μg/ml BSA in HBSS. When Ab was used to block cell surface β-glucan, it was applied to wells for 20 min before addition of PMN. Ab was not washed out to prevent loss of hyphae due to agitation. Under all conditions, 3×10^5 PMN in HBSS was added to each well for a total reaction volume of 200 μl/well, and plates were incubated for an hour at 37°C. Fluorescence was measured using an FL500 Microplate Fluorescence Reader (Bio-Tek Instruments) at excitation/emission wavelength settings of 485/530 nm and a sensitivity setting of 35–40. Results were calculated as the difference in measured fluorescence at 0 min subtracted from that at 60 min and shown as the mean of quadruplicate wells $\pm$ SD.

Adhesion assays

Cells were suspended in HBSS at 5×10^6 cells/ml. CFSE was added to the cells at a final concentration of 10 μM and incubated at room temperature for 20 min in the dark. Cells were washed twice at 4°C, suspended to 3×10^6/ml in HBSS supplemented with 0.3 U/ml aprotinin, 0.05% BSA and 20 μg/ml polymyxin B sulfate, and kept on ice until use. Ab inhibition and SB203580 (1.25 μM) treatment of PMN were performed by the same methods as in the oxidative burst assay. Adhesion was quantified by measuring the fluorescence in each well before and after washing unbound cells. Fluorescence was measured as above. Percent adhesion was calculated as (fluorescence after washing/fluorescence before washing) $\times$ 100. Samples were determined in quadruplicate and results are presented as mean $\pm$ SD.

Confocal microscopy

Glass coverslips were incubated overnight in a solution of 0.5 mg/ml β-glucan or dextran diluted in PBS then blocked with 10 μg/ml PBS for 1 h before adding cells. BSA was removed and immediately replaced by 1×10^5 PMNs suspended in 2 ml Liebovitz's L-15 medium with 2 mg/ml glucose. After incubation for 45 min at 37°C, 2 ml of 2$\times$ formalin was added for 10 min to fix cells. Formalin solution was removed with aspiration and coverslips were gently rinsed then stored in PBS until use. Staining of adhered cells was as follows: block with 10% Fc Block (Accurate Scientific) and 10% serum in PBS for 30 min at room temperature; aspirate solution and add primary Ab mixture of ICRF44 and LM2/1, diluted 1/20, in PBS with 10% Fc Block and 1% FBS, for 60 min at room temp. After washing with PBS, a secondary Ab solution, consisting of Texas red conjugated goat anti-mouse IgG diluted 1/20 into PBS with 1% serum, was applied for 30 min at room temperature. Confocal images were acquired with a Nikon PCM 2000 (Nikon) using green Helium-Neon (excitation 543) laser. Serial optical sections were performed with Simple 32, C-imaging computer software (Compix). Z-series sections were collected at 0.2 μM with a $\times$60 PlanApo lens and a scan zoom of $\times$2. Images were processed using NIH Image 1.62. Image analysis of total fluorescence was measured with AutoQuant software (Meyer Instruments).

Live cell imaging

Delta T glass bottom (Bioptechs) dishes were prepared for hyphae culture by coating with 50 μg of fibronectin diluted in 1 ml of water and incubated

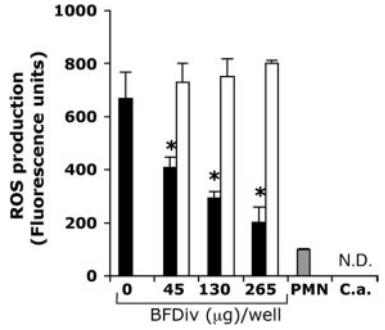

FIGURE 2. Inhibition of *C. albicans*-induced ROS production by β-glucan Ab. Representative graph of three experiments showing PMN production of ROS in response to stimulation with *C. albicans* hyphae (C.a.) that had been preincubated with indicated amounts (per well) of BFDiv Ab (■) or isotype IgM control Ab (□). ▨, PMN response in the absence of hyphae. Each data point reflects the average of six replicate wells ± SD (N.D., not detectable). Significant differences were observed with all BFDiv treatments (*, $p < 0.01$) with respect to isotype control.

overnight. Solution was removed by aspiration, and dishes were gently rinsed once with PBS. *C. albicans* blastoconidia were prepared as described above then 1×10^5 organisms were seeded into dishes in 1 ml of Medium 199. An hour before use, 100 μl of 1 mg/ml BSA was added to hyphae and gently swirled and incubated at room temperature. Supernatant was aspirated and carefully replaced with 1 ml of Lieowitz's L-15 buffer

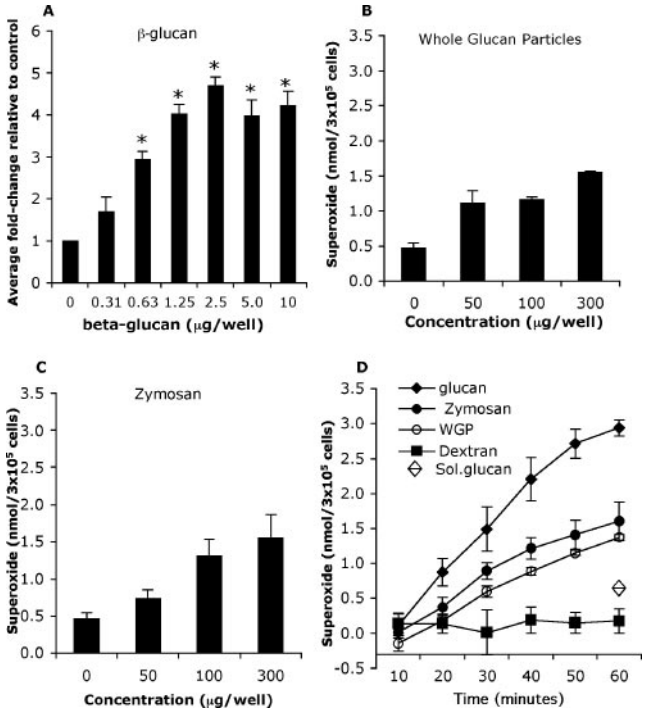

FIGURE 3. Oxidative burst of PMNs in response to immobilized β-glucan, WGPs, or dextran. *A*, PMNs were measured for superoxide (O_2^-) production with increasing amounts of immobilized β-glucan. Change in O_2^- production at 60 min was calculated by normalizing to immobilized dextran controls at each concentration tested. Indicated values represent the mean of 4 donors ± SEM; *, $p < 0.05$ vs dextran. *B* and *C*, O_2^- production at 60 min in response to stimulation with indicated amounts of WGPs (*B*) and zymosan (*C*). *D*, Comparison of O_2^- production over time with: 2.5 μg/well immobilized β-glucan (♦) or dextran (■); 300 μg of zymosan (●) or WGPs (⊖); and 50 μg/ml soluble β-glucan (◇ with line) added directly to wells. Results in *B–D*, the average of four replicate wells ± SD and are representative of at least four experiments. All treatments induced significant O_2^- with respect to dextran or soluble β-glucan ($p < 0.05$).

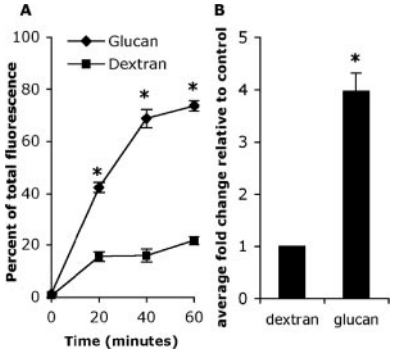

FIGURE 4. β-Glucan promotes PMN adhesion. Fluorescent CFSE-labeled PMNs were added to triplicate wells containing 2.5 μg of immobilized β-glucan, or dextran as a control for nonspecific adhesion. Plates were maintained at 37°C throughout assays and adhesion was measured as described in *Materials and Methods*. A total of 200 nM PMA (data not shown) was used as a positive control for adhesion (100%). *A*, In this representative experiment, values indicate the average remaining percentage of total cellular fluorescence originally added to each of three wells ± SD (*, $p < 0.05$). *B*, For all experiments, change in adhesion at 60 min was calculated with respect to immobilized dextran controls; indicated values represent the mean of six donors ± SEM. *, $p < 0.01$.

supplemented with 2 mg/ml glucose. After placing dish onto FCS2 live cell imaging chamber (Bioptechs), ~20,000 freshly isolated PMNs were added. Events were monitored with Nikon TE 2000-U inverted microscope, images captured with MetaVue acquisition software (Universal Imaging Group). Time-lapse movies were taken every 5 s.

Data presentation

Representative results were obtained from independent experiments conducted three to six times, and values presented are mean ± SD of three to six wells. When not shown in the Figures, the bars depicting the magnitude of the SD were smaller than the symbol. Values combined from multiple experiments are shown as mean ± SEM as indicated. Statistical analysis was performed using ANOVA-Newman-Keuls. The null hypothesis was rejected if $p < 0.05$.

Results

β-glucan is immunodetectable on living C. albicans

Experiments were performed to determine whether β-glucan is immunodetectable on the surface of live fungi. *C. albicans* were grown in culture to the blastoconidial and filamentous forms and incubated with β-glucan-specific Ab (BFDiv), followed by detection with FITC-anti-mouse IgM. Indirect immunofluorescence microscopy showed hyphae to have strong but nonuniform β-glucan staining along the length of filaments (Fig. 1*A*), whereas the cell walls of blastoconidia were weakly fluorescent except for distinct, intensely stained patches, often visible at junctions of emerging buds (Fig. 1*E*). No reactivity was observed with nonspecific murine IgM as primary Ab (Fig. 1*C*). Also, adsorption of BFDiv with excess soluble β-glucan, but not with mannan, inhibited Ab from binding to intact yeast filaments as observed by loss of immunoreactivity (data not shown). The serologic detection of β-glucan indicates that β-glucan is indeed present on the yeast filaments and accessible to Ig.

To verify the specificity of BFDiv for β-glucan, the Ab was adsorbed with polysaccharides of various structural linkages then analyzed for the ability to bind immobilized β-glucan by ELISA. The results in Fig. 1*G*, show that BFDiv binding to β-glucan was inhibited by soluble β-glucan in a dose dependent manner but not by mannan (α1-6 mannose), chitin (β1-4 *N*-acetylglucosamine) or dextran (α1-6 glucose). BFDiv was also used to stain yeast in formalin-fixed, paraffin-embedded kidney sections obtained from

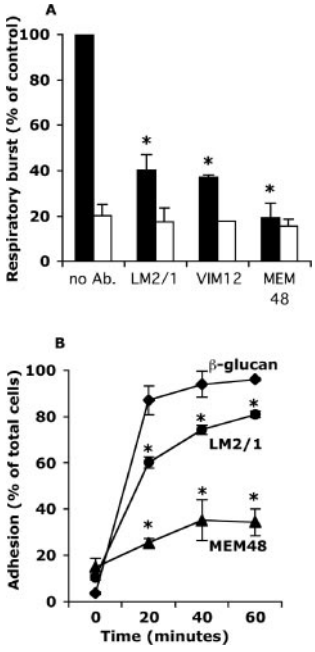

FIGURE 5. Inhibition of CR3 with Ab reduces β-glucan-specific adhesion and superoxide production. PMNs were incubated with anti-CD11b (LM2/1: 5 $\mu g/10^6$ cells and VIM12: 20 $\mu g/10^6$) and anti-CD18 (MEM48: 5 $\mu g/10^6$ cells) Abs for 20 min before adding to wells containing 2.5 μg of immobilized polysaccharide. *A*, Superoxide production with respect to no Ab treatment; ■, β-glucan, and □, dextran. *B*, Adhesion to β-glucan: (♦) β-glucan only; (●) LM2/1; and (▲) MEM48. No effects were observed using IgG as control (data not shown). Values are represented as a percentage of total cellular fluorescence originally added to each well. All results are representative of at least four experiments with three to six replicate wells per sample. ∗, $p < 0.05$ vs immobilized β-glucan alone.

rats that had been infected with *C. albicans.* In this context of in vivo infection, BFDiv Ab bound to the surface of invading hyphae (Fig. 1*H*), similar to what was seen in yeast grown in vitro. Coordinate staining of the infected kidney sections with RP3, an Ab specific for PMN epitopes, revealed the robust response of host PMNs to the site of infection. Evidence for the affinity of PMNs for hyphae was observed ex vivo where PMNs were incubated with unopsonized hyphae and monitored with an image-capturing microscope. The images in Fig. 1*I* were acquired over real time and show a neutrophil approaching a yeast filament, then upon contact, wrapping itself around the hypha as though trying to engulf it completely. However, due to the large size of the hyphae, it is apparent that phagocytosis is frustrated.

To test the hypothesis that β-glucan on fungal filaments is recognized by human PMNs, *C. albicans* hyphae were incubated with several concentrations of BFDiv Ab, or control IgM, to block exposed β-glucan on the fungal surface. Data in Fig. 2 show that PMN production of reactive oxygen stimulated by unopsonized yeast filaments was inhibited with BFDiv in a dose-dependent manner. No significant change was observed in the PMN response to hyphae treated with IgM isotype control Ab.

β-Glucan induces respiratory burst and adhesion in human neutrophils

Experiments tested whether recognition of β-glucan is alone sufficient to induce the generation of O_2^- from neutrophils in the absence of phagocytosis. To approximate the interaction of PMNs with fungal hyphae, freshly isolated cells were plated on increasing concentrations of β-glucan covalently linked to plastic. Superoxide anion production was measured using ferrous cytochrome *c* reduction assays. Immobilized β-glucan induced a dose-dependent respiratory burst in human PMNs (Fig. 3*A*), with a maximal response at concentrations greater than 2.5 μg/well. No reactivity was found to immobilized dextran, mannan, or to β-glucan in soluble form.

Zymosan particles contain a mixture of β-glucan and mannan and are known to activate proinflammatory macrophage functions. To place the efficiency of the respiratory burst to immobilized β-glucan in context, PMNs were stimulated with unopsonized zymosan or whole β-glucan particles (WGPs), which are devoid of mannan. Although WGPs have been stripped of complex yeast cell wall architecture, β-glucan in particulate, phagocytosable form retains a similar profile to zymosan in kinetics and magnitude of the PMN respiratory burst (Fig. 3, *B* and *C*). By comparison, as shown in Fig. 3*D*, the nonphagocytic respiratory burst to immobilized β-glucan was relatively efficient, as ~100-fold less ligand was able to elicit a burst of greater magnitude than that achieved by the particulate stimulants.

To establish a functional correlation between cell adhesion and respiratory burst, neutrophils were plated on 2.5 μg/well immobilized β-glucan (shown in Fig. 3*A* to stimulate maximal respiratory burst). Results in Fig. 4 show that immobilized β-glucan prompted and sustained neutrophil adhesion for the 60 min duration of the experiment. Dextran, which failed to induce a respiratory burst, demonstrated only transient adhesion that could not be supported for longer than 20 min. Taken together, these findings demonstrate that β-glucan supports prolonged adhesion of neutrophils and suggest that β-glucan-induced adhesion may be an underlying mechanism for the oxidative response.

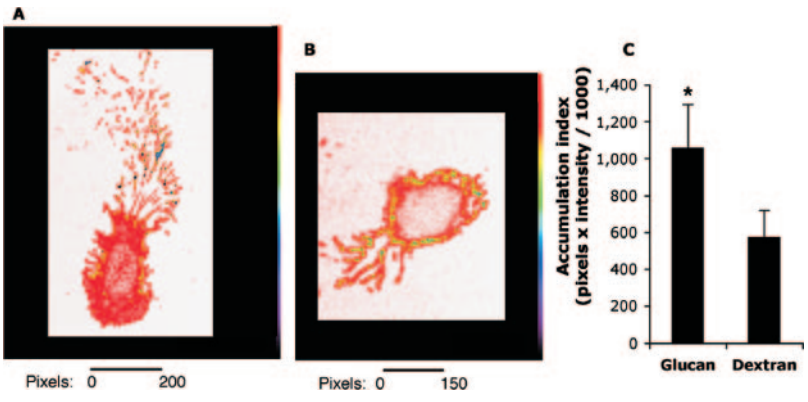

FIGURE 6. CR3 is widely distributed over PMN surfaces adhered to β-glucan. PMNs were allowed to adhere to β-glucan- or dextran-coated coverslips for 30 min then fixed and stained with Abs specific for CR3 (a mixture of ICRF44 and LM2/1). Confocal images represent PMNs adhered to β-glucan (*A*) or to dextran (*B*). Plane of focus is between cell and substrate. CR3 staining intensity is defined in color bars to the right of each image (red is the highest in range) and quantified in *C*. ∗, $p < 0.05$ vs dextran.

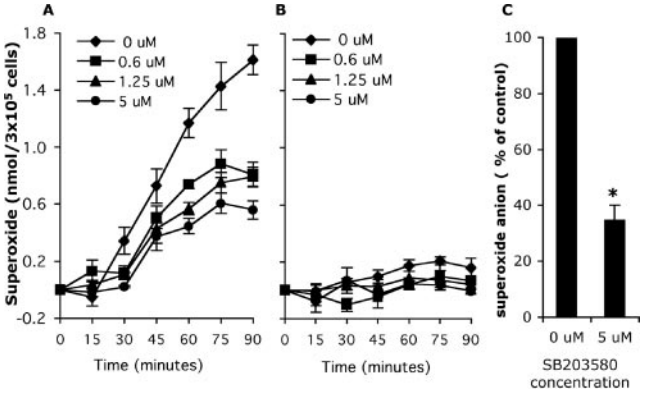

FIGURE 7. Inhibition of p38 MAPK activity reduces β-glucan-mediated respiratory burst. PMNs were incubated with the indicated concentrations of SB203580 for 10 min before addition to 2.5 μg/well immobilized β-glucan (*A*) or dextran (*B*). Superoxide production was measured kinetically for 90 min. Values are the average ± SD of quadruplicate wells and results are representative of four experiments. Differences were significant from 30 to 90 min in all treatments with respect to β-glucan alone ($p < 0.05$). *C*, For all experiments, the percentage of change in O_2^- production with 5 μM SB203580 was calculated at 60 min by normalizing against untreated or DMSO controls. Indicated values represent the mean of four donors ± SEM. ∗, $p < 0.01$.

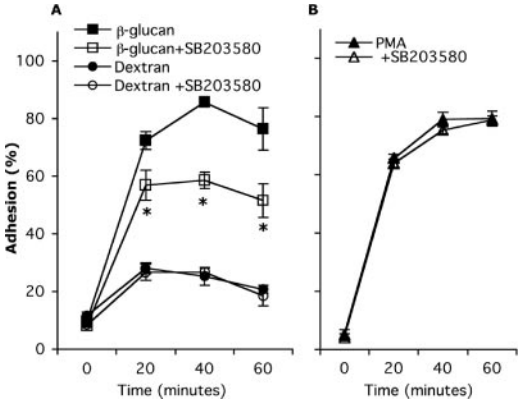

FIGURE 8. Inhibition of p38 MAPK activity reduces adhesion to β-glucan. Fluorescent CSFE-labeled PMNs were incubated with 1.25 μM SB203580 for 10 min before being added to wells containing 2.5 μg of immobilized β-glucan or dextran (*A*) or 200 nM PMA (*B*). Values are represented as a percentage of total fluorescence of triplicate wells. ∗, $p < 0.05$ with respect to immobilized β-glucan alone.

CR3 is required to stimulate β-glucan-specific neutrophil adhesion and superoxide release

Because CR3 has been shown to function as a receptor for β-glucan in neutrophils (22, 24, 25), we sought to determine its role in the adhesion-dependent respiratory burst to immobilized β-glucan. Abs against the CD11b and CD18 subunits of CR3 (LM2/1, VIM12, and MEM48), respectively, were used in blocking experiments. Results in Fig. 5*A* show that MEM 48 was able to reduce the β-glucan induced burst to background levels, while VIM12, which has been shown to block β-glucan binding (13, 24), and LM2/1 suppressed the burst by 50% or more. Inhibition of CD18 appeared to abrogate the β-glucan response most effectively since treatment of PMN with IB4, another CD18 Ab, yielded results very similar to MEM48 treatment (data not shown). Also, the burst response was unaffected by PMN treatment with IgG as an Ab control.

To test the function of CR3 in PMN adhesion to β-glucan, cells were treated with MEM48 or LM2/1 Abs and measured for their capacity to adhere to β-glucan (Fig. 5*B*). In direct correlation with findings for respiratory burst activity, MEM48 diminished adhesion to levels near background, while LM2/1 blocked adhesion by 17–30% over the course of 60 min.

Images obtained using confocal microscopy further attest to the relevance of CR3 for PMN adhesion to β-glucan. Cells attached to coverslips coated with either dextran or β-glucan were stained with anti-CR3 Abs. Images taken at the plane where cells interface with the matrix surface revealed that PMNs were broadly spread on β-glucan and showed intense staining for CR3 throughout a wide margin of the cell perimeter (Fig. 6*A*). In contrast, cells adhered to dextran appeared to have CR3 staining in a comparatively thin peripheral margin (Fig. 6*B*) and overall staining was significantly less than on cells adhered to β-glucan (Fig. 6*C*).

β-Glucan activation of PMN requires p38 MAPK

Several reports indicate a role for p38 MAPK in mediating the adhesion-dependent respiratory burst, particularly when leukocyte integrins such as CR3 have a regulatory role (27, 38). To test whether p38 MAPK was required for the oxidative burst response to β-glucan, neutrophils were treated with a specific inhibitor of p38 MAPK activity (27), SB203580. The inhibitor concentrations tested resulted in a significant inhibition of the β-glucan-induced oxidative burst (Fig. 7) but DMSO, in which the inhibitor was dissolved did not affect this response (data not shown). In addition to the reduced magnitude of respiratory burst output, inhibition of p38 MAPK activity consistently resulted in a delayed onset of burst activity of at least 10 min, thus suggesting a role for p38 MAPK in assembly of the plasma membrane NADPH oxidase complex.

SB203580 was then used to test whether p38 MAPK inhibition could affect PMN adhesion to β-glucan. The results in Fig. 8*A* show that, although SB203580-treated cells were able to initiate adhesion to β-glucan at the same rate as untreated cells, the extent of adhesion was significantly attenuated. The adhesion of cells to dextran (Fig. 8*A*) or of cells stimulated with PMA (Fig. 8*B*) was not affected by inhibition of p38 MAPK and PMN response to β-glucan was not affected by DMSO vehicle control (data not shown). These data demonstrate a role for p38 MAPK in the regulation of PMN adhesion to β-glucan and the consequent respiratory burst.

β-Glucan activation of PMNs does not require PKCζ signaling

PKCζ is an atypical PKC that acts independently of Ca^{2+} and diacylglycerol (39). Previous evidence indicates a role for PKCζ in the regulation of neutrophil integrin-dependent adhesion, chemotaxis

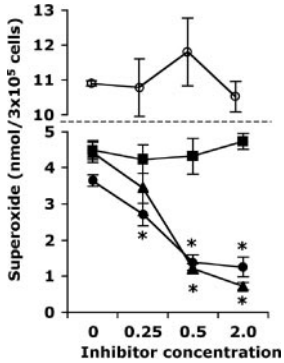

FIGURE 9. PKCζ signaling is not required for β-glucan-induced superoxide production. Cells were treated with a myristoylated PKCζ pseudosubstrate for 10 min before initiation of respiratory burst assays. Agonists were: 2.5 μg of immobilized β-glucan(■), 10^{-6} M fMLP (●), 5 μg/ml sBLP (▲), or 200 nM PMA (○). Superoxide production was measured for 60 min. ∗, $p < 0.05$ vs immobilized β-glucan.

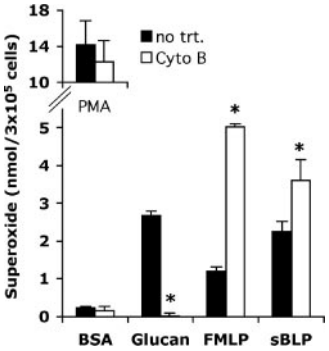

FIGURE 10. Effect of cytochalasin B on the respiratory burst of human neutrophils. Superoxide anion production in response to stimulation with 2.5 μg of immobilized β-glucan, 10^{-6} M fMLP, 5 μg/ml sBLP, or 200 nM PMA was determined in the absence (■) or presence (□) of cytochalasin B for 60 min. Cytochalasin B treatment (2 μg/ml) was significant in all groups except PMA. *, $p < 0.05$ vs untreated.

(40), and respiratory burst (33). Following this evidence, the relevance of PKCζ activity to β-glucan induced PMN function was examined. PKCζ was inhibited using a myristoylated pseudosubstrate peptide containing a sequence that would compete with the endogenous substrate (40). Cells were treated with inhibitor for 10 min before being assayed for β-glucan-induced O_2^- production, compared with that triggered by alternative neutrophil agonists. The results shown in Fig. 9 indicate that PKCζ was not required for rapid O_2^- production in response to immobilized β-glucan, in contrast to the dose dependent inhibition of respiratory burst to fMLP and sBLP. PMA is cell permeable, does not require a receptor, and activates both conventional and novel classes but not atypical PKCs (41). The PKCζ antagonist did not interfere with the burst to PMA, demonstrating specificity of the inhibitory peptide for the atypical ζ isoform.

β-Glucan induced respiratory burst is abolished by cytochalasin B

The use of cytochalasin B to disrupt f-actin is agonist-dependent, as evidenced by its potentiation of O_2^- production to fMLP (42) but inhibition of the response to TNF-α (43). Therefore, experiments were performed to determine the participation of the actin cytoskeleton in mediating the respiratory burst to β-glucan. The results in Fig. 10 show that cytochalasin B completely abolished O_2^- production by β-glucan while augmenting the burst response to alternative PMN stimulants fMLP and the TLR2 ligand sBLP. The importance of the actin cytoskeleton in the PMN response to β-glucan is consistent with an integrin-mediated event.

Discussion

Accessibility of *C. albicans* β-glucan to recognition by the immune system has been a topic of debate, because the vast majority of β-glucan is complexed with chitin and lies beneath a layer of mannoprotein (44). Using BFDiv, a mAb specific for β-glucan, our experiments show that β-glucan is clearly detectable, in both a distinct focal pattern on the cell walls of *C. albicans* blastoconidia (Fig. 1*E*) and abundantly distributed throughout the length of hyphal filaments (Fig. 1*A*). The punctate staining seen in the yeast form is likely due to cell wall remodeling from the budding process, and would presumably increase with the progressive events of proliferation. The conspicuous association of β-glucan staining pattern with the morphologic form of *C. albicans* has also been observed by others who used another β-glucan specific Ab unrelated to BFDiv (45). In contention with these collective observations though, Gantner et al. (8) reported that β-glucan could not be detected in the hyphal cell wall with recombinant soluble Dectin-1, the main receptor used by murine macrophages and dendritic cells to recognize β-glucan (46). The differences in findings may be due to affinity, protein conformation or molecular features within the epitopes, or may apply more broadly to serologic vs receptor-mediated immune mechanisms. Nonetheless, the overall ability of Ab to bind β-glucan as a feature of the hyphal surface is particularly useful for experimental blocking protocols.

Human neutrophils respond avidly to the presence of *C. albicans*, illustrated by their abundance in infected tissue and in ex vivo experiments (Fig. 1, *H* and *I*). Our studies show that adsorption of hyphae with BFDiv to block β-glucan was effective in reducing the respiratory burst of human PMNs to filaments. Although recognition of *C. albicans* in vivo is undoubtedly a complex process, this data suggests that β-glucan on the surface of hyphae directly stimulates a biological response in PMNs.

The production of ROS is vital to the role of PMNs in host defense. The microbicidal properties of ROS include damage to fungal cells and hyphae and animals with defects in ROS production are highly susceptible to fungal challenge (47). The apparent relationship we observed between ROS production and cell wall β-glucan indicates that the latter is an element of invading *C. albicans* that acts to distinguish the potential pathogen to the immune system. This concept was the motive for investigating both β-glucan as a constituent mechanism for PMN recognition of the hyphal form of fungi, and the pathways leading to the production of ROS. To examine how β-glucan could affect neutrophil function in isolation from other cell wall components, an in vitro assay system was used in which β-glucan was covalently coupled to a plastic substratum, allowing PMNs contact with β-glucan but impairing uptake. This process has been described as frustrated phagocytosis (48) and is evident in images of PMN envelopment but not engulfment of hyphae (Fig. 1*I*).

Under these conditions, immobilized β-glucan alone was sufficient to stimulate rapid adhesion of human neutrophils and to induce a robust and sustained respiratory burst that was concentration dependent (Figs. 3 and 4), demonstrating that β-glucan in this form is able to act as both an adhesion anchor and an agonist of O_2^-. We observed no induction of O_2^- by stimulation with immobilized mannan, consistent with a report that showed respiratory burst induced by phagocytosis of zymosan was due to its β-glucan content rather than to its mannan content (49). Moreover, our studies showed no significant differences in O_2^- production between zymosan and WGPs (Fig. 3*D*), indicating that β-glucan does not lose its potency when separated from other yeast components. Fig. 3*D* also shows that β-glucan in soluble form does not elicit respiratory burst activity, while immobilization allows for adhesion-dependent respiratory burst. Although the mechanism for the differential response to immobilized vs soluble β-glucan is not known, it is plausible that immobilization allows for the receptor cross-linking and/or clustering necessary to induce an oxidative response. By requiring multifocal contact for respiratory burst, neutrophils extravasating to fungally infected tissues may be prevented from releasing oxidative radicals until tightly bound to hyphae.

The receptors for β-glucan that have been studied extensively are the integrin CR3 (CD11b/CD18), Dectin-1, (46) and its human homologue βGR (4). β-glucan activation of CR3 is thought occur via the lectin like domain on the receptor (13, 19). The ability of CR3 ligation to signal a respiratory burst response (50, 51) is somewhat controversial. Activation by adhesion to its more conventional ligands (ICAM, fibrinogen, C3bi) is a known prerequisite for initiating respiratory burst to soluble cytokines (21), but in contrast, others demonstrated that neither activation of CR3 by its complement ligand, iC3b (52) nor by Ab-mediated cross-linking

(53) could induce significant release of ROS by human PMNs. We were interested to know whether CR3 was important in the PMN response to the immobilized form of β-glucan. In our experiments, blocking CR3 with Ab significantly decreased PMN adhesion and superoxide production to immobilized β-glucan (Fig. 5), suggesting that the availability of CR3 is essential for PMNs to respond optimally to β-glucan. Further support for the importance of CR3 was seen in confocal images that showed accumulation of CR3 to be significantly higher and more widely distributed at the interface of PMNs adhered to β-glucan than cells on dextran (Fig. 6). Since Ab blocking of CR3 did not completely inhibit PMN responses, it is likely that other receptors cooperate with CR3 to induce anti-β-glucan activity. Dectin-1 and βGR have been characterized almost exclusively with regard to macrophage and dendritic cell responses, although human neutrophils have been shown to express the receptor (4). There is little evidence of the receptor's activity in neutrophils, and a recent study has shown that human granulocytes from an in vivo inflammation model actually decreased expression of surface βGR (5). The possibility that CR3 and βGR or other receptors may coordinate PMN responses to β-glucan of has not yet been ruled out and requires further investigation.

Activation of p38 MAPK has been shown to play a central role in maximal neutrophil responses to cytokines and bacterial peptide agonists (27, 29, 54). However, the requirement for p38 MAPK does not appear to be consistent among activated neutrophil functions and appears to be agonist dependent (27, 29, 55). We sought to determine the importance of p38 in signaling β-glucan-induced functions and show that blockade of p38 MAPK activity moderately but significantly decreases both adhesion and the oxidative burst response to β-glucan (Figs. 7 and 8). The events downstream of p38 MAPK that regulate the adhesion-dependent respiratory burst have not yet been elucidated. With regard to adhesion, it is known that p38 MAPK activation is associated with actin binding proteins involved in cytoskeletal rearrangement (30, 31, 56). Thus, the upstream inhibition of p38 may indirectly inhibit the activity of these proteins to prevent maximal cell adhesion. Alternately, SB203580 has previously been shown to inhibit CR3-containing vesicles from fusing with the cell surface in TNF-α-stimulated cells (38, 57), suggesting that decrease in available receptor number might account for the decreased adhesion we observed, while existing surface CR3 receptors are available to mediate some degree of function. p38 independent signaling mechanisms of PMN adhesion to β-glucan will be the focus of future investigation.

Given the known correlation between adhesion and burst functions (21, 27), the reduced adhesion resulting from p38 MAPK inhibition (Fig. 8) would seem sufficient to explain the similar reduction of the oxidative burst (Fig. 7). However, p38 may function in dual roles to signal both adhesion and NADPH oxidase assembly, because it was demonstrated to directly phosphorylate cytosolic oxidase components $p47^{phox}$ and $p67^{phox}$ in neutrophils (55). Although we have not established a specific function for p38 MAPK, it is clear that β-glucan may be included among the agonists that require p38 MAPK to generate maximal O_2^- release from PMN.

Adhesion-dependent activation of the respiratory burst has been reported to involve multiple integrin signal transduction pathways (as reviewed in Ref. 58). PKCζ was considered as a candidate in β-glucan signaling based on previous reports showing that it is able to phosphorylate and activate $p47^{phox}$ (33) in fMLP-stimulated PMNs and to participate in PMN adhesion (40). However, activation of PKCζ, like p38 MAPK, may be agonist-dependent, as our studies found that it did not have a role in signaling the β-glucan-induced respiratory burst.

The inclusion of the authentic TLR-2 ligand Pam_3CSK_4 (sBLP) in our studies demonstrates the ability of TLR-2 ligation to induce a respiratory burst in human PMNs, in agreement with another report (59). The qualitative differences we observed in sensitivity to PKCζ inhibition and to cytochalasin B suggests that TLR-2 and CR3 do not share these intracellular signaling pathways, at least in vitro, and thus are not cooperative in triggering the nonphagocytic respiratory burst to immobilized β-glucan.

Sensitivity to cytochalasin B indicates that an intact cytoskeleton is required to manifest β-glucan-dependent respiratory burst (Fig. 10), similar to agonists such as TNF-α, IL-8, and platelet activating factor (42). Interestingly, actin function is also required for phagocytosis of *C. albicans* blastoconidia, germ tubes, and short filaments (60). Although the actin cytoskeleton is believed to provide a means for coordinating the process of oxidase assembly (61), its function is not required for all agonists, as observed with fMLP and sBLP (Fig. 10). The particular signaling requirements of cytoskeleton and p38 but not PKCζ may reflect a signaling architecture within human PMNs that is unique to the response to β-glucan.

In summary, this report shows that β-glucan is accessible to the cells of the human immune system responsible for initial and innate host defense against infection. We have demonstrated that β-glucan can be detected serologically by specific Ab in both the cellular and hyphal forms of *C. albicans* and is directly recognized in filaments by PMNs. When used in in vitro assays, immobilized β-glucan was both anchor and agonist, sufficient to induce rapid human PMN adhesion and superoxide production. p38 MAPK but not PKCζ, was required signaling for maximal PMN responsiveness. The integrin CR3 is a key mediator of the PMN response to β-glucan, expanding the role of this receptor role to include nonopsonic recognition of a pathogen-associated molecule to induce host defense.

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Disclosures

The authors have no financial conflict of interest.

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