The effect of PGG-β-glucan on neutrophil chemotaxis in vivo

Brian W. LeBlanc, Jorge E. Albina, and Jonathan S. Reichner[1]

Department of Surgery, Rhode Island Hospital and Brown Medical School, Providence

Abstract: The β-glucans are long-chain polymers of glucose in β-(1,3)(1,6) linkages, which comprise the fungal cell wall and stimulate cells of the innate immune system. Previous in vitro studies have shown the ability of β-glucan to increase the chemotactic capacity of human neutrophils. The current study examined an in vivo correlate of that observation by testing the hypothesis that systemic β-glucan treatment would result in enhanced migration of neutrophils into a site of inflammation and improve antimicrobial function. A model of acute inflammation was used in which polyvinyl alcohol sponges were implanted subcutaneously into the dorsum of rats. Animals treated with β-glucan showed a 66 ± 6% and 186 ± 42% increase in wound cell number recovered 6 and 18 h post-wounding, respectively. Increased migration did not correlate with increased chemoattractant content of wound fluid, alterations in neutrophil-induced loss of endothelial barrier function, or changes in neutrophil adhesion to endothelial cells. Systemic administration of SB203580 abrogated the enhanced migration by β-glucan without altering normal cellular entry into the wound. Studies also showed a priming effect for chemotaxis and respiratory burst in circulating neutrophils isolated from β-glucan-treated animals. Heightened neutrophil function took place without cytokine elicitation. Furthermore, β-glucan treatment resulted in a 169 ± 28% increase in neutrophil number and a 60 ± 9% decrease in bacterial load in the bronchoalveolar lavage fluid of *Escherichia coli* pneumonic animals. Taken together, these findings demonstrate that β-glucan directly affects the chemotactic capacity of circulating neutrophils through a p38 mitogen-activated protein kinase-dependent mechanism and potentiates antimicrobial host defense. *J. Leukoc. Biol.* 79: 667–675; 2006.

Key Words: *priming · host defense · rat · p38 MAPK*

INTRODUCTION

Rapid recruitment of circulating polymorphonuclear leukocytes (PMNs; neutrophils) into a site of injury is essential for mounting an effective inflammatory response. The migratory process of these early-response, innate immune cells is the result of a well-orchestrated sequence of activation and adherence events [1]. Neutrophils tether along the vascular endothelium via selectins, followed by firm adhesion using β_2-integrins, and β_2-integrin ligation signals the activated neutrophil to secrete mediators that alter endothelial barrier function, resulting in vascular permeability and neutrophil transmigration [2, 3]. Following extravasation, neutrophils migrate through the extracellular matrix (ECM) of the injured tissue toward local chemokines by transient integrin interactions with ECM components [4].

Although the innate immune system has been studied for a number of years, a well-defined pharmacological intervention to improve the function of neutrophils or macrophages is still lacking. This is so, as the favorable aspects of cell priming needed to improve host defense are often accompanied by the overproduction of proinflammatory mediators. The β-glucans are a class of long-chain polymers of glucose in a β(1,3)(1,6) linkage, which are a structural component of the fungal cell wall. These molecules are not found in mammalian cells and are considered pathogen-associated molecular patterns, which are recognized by the innate immune system of vertebrates and invertebrates [5]. β-Glucan extracted from *Saccharomyces cerevisiae* has been shown to potentiate leukocyte functions and not elicit cytokine production in experimental animals, human whole blood, or monocytic cell lines [6–9]. Furthermore, β-glucan did not exhibit pyrogenic properties in humans at therapeutic doses [10, 11].

The demonstration that it is possible to augment innate immune cell functions separately from cytokine production, prompted this laboratory to further characterize the mechanism of action of β-glucan, given its therapeutic potential. In vitro findings identified a dose-dependent, promotional effect of β-glucan on human PMN chemotaxis toward formyl-Met-Leu-Phe (fMLP) and C5a [12, 13]. In contrast and consistent with an anti-inflammatory effect, β-glucan reduced directional migration toward interleukin (IL)-8 and maintained endothelial barrier function in the presence of activated neutrophils [13, 14]. Given the opposing effect of β-glucan on chemotaxis toward individual chemokines in vitro, the aim of this study was to determine the effect of β-glucan on the migration of peripheral blood PMNs (pbPMNs) into a site of sterile injury, where the response is mediated by host-derived chemoattractants. Findings to be shown reveal a significant increase in neutrophil infiltration into a site of local inflammation following

[1] Correspondence: Division of Surgical Research, Rhode Island Hospital, NAB 219, 593 Eddy Street, Providence, RI 02903. E-mail: Jonathan_Reichner@Brown.edu

Received March 13, 2005; revised November 3, 2005; accepted November 17, 2005; doi: 10.1189/jlb.0305150.

β-glucan treatment, which is reduced to normal levels by systemic inhibition of p38 mitogen-activated protein kinase (MAPK). In light of the multistep nature of neutrophil extravasation, subsequent studies were performed to identify specific aspects of the process affected by β-glucan treatment. With respect to its therapeutic potential, the ability of β-glucan to improve antimicrobial host defense was demonstrated by a significant leukocytic response to bacterial pneumonia and reduction of bacterial burden.

MATERIALS AND METHODS

Animals

Male Fisher-344 rats (150–200 g; VAF-plus, Charles River Laboratories, Wilmington, MA) were housed in barrier cages and fed rat chow and water ad libitum. VAF-plus rats were certified free of common rat pathogens by the supplier and were monitored by Brown University/Rhode Island Hospital (Providence) veterinary personnel.

Sterile wound model

The subcutaneously (s.c.) implanted polyvinyl alcohol (PVA) sponge wound model was described previously [15]. Briefly, 10 sterile square sponges ~1 cm^2 and 0.25 cm in thickness (PVA Unlimited, Warsaw, IN) were implanted s.c. in the dorsum of anesthetized rats by inhalation of 3% isoflurane (Abbott Laboratories, North Chicago, IL) through a 3-cm midline incision. The sponges were retrieved 6 or 18 h after wounding and placed in 20 ml Hanks' balanced saline solution (HBSS; Invitrogen Corp., Carlsbad, CA). The cells contained in the sponges were isolated by repeated rapid compression using a Stomacher (Tekmar Co., Cincinnati, OH) for 30 s, centrifuged at 700 *g* for 10 min at 4°C, and resuspended in Dulbecco's phosphate-buffered saline (dPBS; Invitrogen Corp.). Histological examination and DNA quantification of the sponge material after cell extraction indicated complete cell recovery. Cells obtained in this manner are >95% viable according to trypan blue exclusion. Absolute numbers of wound cells were calculated with the aid of a hemacytometer and from differential counts after staining cytospins (~10^5 cells per slide) with hydroxyethyl methacrylate (HEMA) 3 (Fisher Scientific, Pittsburgh, PA). Wound fluid was obtained by placing the retrieved sponges into a 5-ml syringe supported in a 15-ml conical centrifuge tube and centrifuged at 700 *g* for 10 min at 4°C. Cell-free wound fluid was frozen at −80°C until use.

Purified β-glucan injection

The highly purified, pharmaceutical grade β-glucan used in this study was originally prepared by Alpha-Beta Technology, Inc. (Worcester, MA) and referred to as poly-β1-6-glucotriosyl-β1-3-glucopyranose glucan or BETAFECTIN™. It was provided to our laboratory by Biothera (Eagan, MN) and referred to as β-glucan in this report. A single bolus of 2 mg/kg-purified β-glucan, molecular weight (M.W.) 150 ± 20 kDa, or dextran, M.W. ~78 kDa (Sigma Chemical Co., St. Louis, MO), was injected intravenously (i.v.) 15 min prior to sponge implantation.

p38 MAPK activity inhibition in vivo

Rats were injected intraperitoneally (i.p.) with 20 mg/kg SB203580 hydrochloride (Tocris Cookson Inc., Ellisville, MO) 30 min prior to wounding as above described.

p38 MAPK Western blot

Freshly isolated rat neutrophils (see below) were washed twice with F-12K media (Invitrogen Corp.) and resuspended in F-12K media supplemented with 20% fetal calf serum. Cells were placed at 37°C for 30 min, and then prewarmed β-glucan (10 μg/ml) was added without mixing. At each time-point, a fivefold volume of ice-cold dPBS was added and immediately centrifuged at 12,000 *g* for 10 s. Supernatant was removed, and the cell pellet was snap-frozen in liquid nitrogen and stored at −80°C. Cell pellets were resuspended in ice-cold modified radio immunoprecipitation assay buffer [50 mM Tris-HCl at pH 7.4, 1% Nonidet P-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 1 mM NaF, and Complete protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN)], to which was added an equal volume of sodium dodecyl sulfate (SDS) loading buffer supplemented with 2-mercaptoethanol, followed by sonication and boiling 10 min. The proteins were separated on a 10% SDS-polyacrylamide gel (Invitrogen Corp.) and electroblotted on nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA.) Membranes were blocked with 5% dry milk (Bio-Rad Laboratories) and probed with antiphospho p38 MAPK (Thr180/Tyr182; 1:2000; #9216S, Cell Signaling Technology, Beverly, MA) and anti-mouse immunoglobulin G [IgG; horseradish peroxidase (HRP)-linked; 1:2000, #7072-1, Cell Signaling Technology), followed by luminescence detection according to the manufacturer's protocol (enhanced chemiluminescence, Amersham Life Technologies, Arlington Heights, IL). Membranes were stripped and reprobed with anti-p38 MAPK antibodies (1:1000; #9212, Cell Signaling Technology) and anti-rabbit IgG (HRP-linked; 1:2000, #7074, Cell Signaling Technology.) Gels were scanned using an EPSON 4180, and protein bands were quantified by densitometry using ImageJ (v1.34 s, National Institutes of Health, Bethesda, MD).

pb Neutrophil isolation

Whole blood was obtained by puncture of the inferior vena cava (IVC) from 18-h wounded animals or 18-h β-glucan treatment. Red blood cells were lysed with hypotonic saline and resuspended in dPBS. Cells were isolated using a biotinylated mouse anti-rat PMN IgG at 1:250 dilution (clone RP3) [16] with Fc-Block (Accurate Chemical, Westbury, NY) to block nonspecific binding. Cells were incubated 30 min on ice and washed, and antibiotin microbeads (Miltenyi Biotec, Auburn, CA) were added to the cells, incubated 30 min on ice, washed twice with dPBS, and placed on Miltenyi mass spectroscopy magnetic bead separation columns. Isolated cells were >95% viable by trypan blue exclusion and were >90% PMNs.

Chemotaxis assay (modified Boyden chamber)

Wound or blood cells (3×10^6 cells/ml dPBS) were fluoresceinated by the addition of 10 μM carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes, Inc., Eugene, OR) in the dark at room temperature for 30 min, washed twice with dPBS, allowed to stand for 30 min, and then washed twice more with dPBS. Cells (1×10^6)/200 μl dPBS were added to the top of a 3.0-μm pore-size tissue culture-treated polycarbonate membrane 24-well transwell system (Falcon Labware, Becton Dickinson, Franklin Lakes, NJ). Wound fluid (700 μl 10%) in dPBS as the chemotactic source was added to the bottom of the 24-well plate, and cells were allowed to migrate for 30 min at 37°C. Inserts were removed, and fluorescence was read in a FL500 microplate fluorescence reader (Bio-Tek Instruments, Winooski, VT) with excitation/emission wavelength settings of 485/530 nm and a sensitivity setting of 40–44. A standard curve of fluorescent cells was performed for each assay.

Respiratory burst assay

A low-endotoxin bovine serum albumin (BSA; 100 μL 10 mg/ml, Sigma Chemical Co.) solution was added to wells of a high-binding 96-well plate (Corning Inc., Corning, NY), incubated at 37°C for 1 h and washed three times with dPBS. To form immune complexes, 100 μL of a 10 μg/ml anti-BSA antibody (Rockland, Gilbertsville, PA) was added, incubated 37°C for 1 h, and washed three times with dPBS. Negative control wells were treated with BSA only. Production of superoxide (O_2^-) was quantitated by measuring the O_2^- dismutase-inhibitable reduction of ferricytochrome c, as described previously [17]. Wound or blood cells (3×10^5 cells/ well) were resuspended in 200 μL phenol red-free HBSS (Invitrogen Corp.) containing 75 mM horse heart ferricytochrome c (Sigma Chemical Co.). The plates were then incubated at 37°C for 60 min and the absorbance at 550 nm against a reference 630-nm filter, measured every 10 min in an EL340 microplate reader (Bio-Tek Instruments). O_2^- production was calculated from the difference in absorbance at 550 nm (corrected for absorbance at 630 nm) and the extinction coefficient for the absorbance of reduced ferricytochrome c using the equation $\Delta E_{550\ nm-630\ nm} = 2.1 \times 10^4\ M^{-1} \cdot cm^{-1}$.

Electrical impedance measurement

Transendothelial barrier electrical resistance was measured in real time using an electric cell-substrate impedance sensor system (ECIS; Applied Biophysics,

Inc., Troy, NY) [14, 18]. Rat pulmonary arterial endothelial cells (RPAECs) were obtained from Dr. Elizabeth Harrington (Providence VA Medical Center, RI) and grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Corp.) with 20% fetal bovine serum (FBS; Hyclone Labs, Logan, UT) supplemented with antibiotics. Cells, between passages 17 and 24, were plated on sterile, gold-plated electrode arrays, which were precoated with collagen Type I (30 mg/ml, BD Biosciences, Medford, MA) and grown to confluence. After being changed to fresh media, the electrode array was mounted onto holders of the ECIS system housed within an incubator (37°C, 5% CO_2) and connected to a lock-in amplifier. Prior to use, confluence of endothelial monolayers was established on the ECIS arrays by initial reading of the endothelial resistance. Only confluent monolayer reading resistance between 1000 and 1300 Ohms was used in the study. Neutrophils (6×10^5 cells per well in 200 μl serum-free media) isolated from β-glucan-treated or -untreated, 18 h wounded rats were added on each well to give a 3:1 PMN:EC ratio. Thrombin (0.8 unit/ml) is known to cause instant retraction and was used in selected samples as a positive control to ensure that cells respond as expected prior to use in experiments. Monolayer resistance was recorded over 3 h in real time at 1-min intervals after PMN addition. Values are reported as normalized to initial baseline resistance.

Endothelial adhesion assay

RPAECs were plated 5×10^5 cells/well on 96-well tissue-culture plates (Becton Dickinson) precoated with collagen Type 1 (30 mg/ml in PBS) and grown to confluence. ECs were stimulated, or not, with rat tumor necrosis factor α (TNF-α; 50 ng/ml in PBS) for 6 h and then washed three times with sterile PBS. CFSE fluorescent pbPMNs from β-glucan-treated or -untreated, 18 h wounded rats were added 2×10^5/150 μl complete DMEM (Invitrogen Corp.) supplemented with 10% FBS (Hyclone Labs) to each well and incubated for 45 min at 37°C in 5% CO_2. Nonadherent cells were removed by washing twice with serum-free DMEM, and fluorescence was measured using a fluorescent plate reader (above). All determinations were carried out in six-well replicates and repeated three times.

Cytokine and chemokine content determination

Wound fluid was collected as described above, and serum was from whole blood collected via the IVC into untreated glass vacutainer tubes. The blood was allowed to clot for 3 h at 4°C, and serum was separated after 10 min spin at 700 *g*. Wound fluid and serum were frozen at −80°C, and aliquots were sent to Searchlight Multiplex sample analysis service (Pierce Chemical Co., Woburn, MA).

Acute inflammation during *Escherichia coli* pneumonia

To measure the effect of β-glucan on the neutrophilic response to a site of infection, 200 g male Fisher-344 rats were injected with β-glucan (2 mg/kg) i.p., followed by a second application of 2 mg/kg i.v. 18 h later at the time of bacterial instillation. After rats were anesthetized by inhalation of 3% Isoflurane, the trachea was exposed surgically, and 250 μl *E. coli*, suspended to $\sim6 \times 10^8$ colony-forming units (cfu)/ml in 0.9% PBS with 5% colloidal carbon (to mark the site of deposition), was delivered by intratracheal instillation, which was directed into the left lung using an 18-gauge (ga) angiocath (Becton Dickinson). After 6 h, rats were killed by lethal overdose of CO_2. The hearts were tied off to maintain pulmonary blood, and pb was collected via the IVC. The lungs were catheterized using an 18-ga angiocath, lavaged four times using 3 ml ice-cold dPBS, removed, and fixed by the intratracheal instillation of 10% buffered formalin at a pressure of 23 cm H_2O.

Bacterial killing and leukocyte counts after *E. coli* pneumonia

Total bronchoalveolar lavage (BAL) fluid was serially diluted in normal saline, and 100 μl aliquots were spread using a glass rod onto Luria-Bertani agar plates. After an overnight incubation at 37°C, CFU were enumerated. Total lavage cell numbers were obtained using a hemacytometer after dilution in 4% trypan blue (Invitrogen Corp.). Cell morphology was determined by HEMA 3 staining.

Endotoxin

All reagents used contained less than 0.1 EU/ml endotoxin, as determined by *Limulus* amoebocyte lysate screening (BioWhittaker, Walkersville, MD).

Statistics

Data were presented as means ± SE. β-Glucan-treated and control rats were compared by ANOVA with Newman-Keuls or by Mann-Whitney *U*-test as appropriate, and differences were considered significant when $P < 0.05$.

RESULTS

β-Glucan treatment increases cellular response to injury

s.c. implantation of PVA sponges was used to determine whether the β-glucan-induced enhancement of chemotaxis observed in vitro translates into augmented entry of circulating neutrophils into a site of sterile injury. Studies focused on the neutrophil-predominant phase of wound healing, which occurs within 24 h postinjury. **Figure 1** shows a significant increase in total cell number collected from the sponges of β-glucan-treated rats compared with untreated rats at 6 h postwounding ($1.8\pm0.1\times10^7$ vs. $1.0\pm0.1\times10^7$, treated vs. untreated, respectively; $P<0.05$). A further increase was noted in those isolated at 18 h postwounding ($3.5\pm0.9\times10^7$ vs. $1.1\pm0.3\times10^7$, treated vs. untreated, respectively; $P<0.05$). Dextran, a polysaccharide control, showed no significant difference in cell number compared with untreated control animals at 6 and 18 h. Differential staining of wound cells showed the population to be composed of 91.3 ± 1.0% PMNs at 6 h and 91.4 ± 0.5% PMNs at 18 h with no significant difference between β-glucan-treated and -untreated animals (not shown). Macrophages were the predominant remaining cell type at both time-points.

β-Glucan treatment effect on pb neutrophil chemotaxis

To determine whether β-glucan treatment directly affects the chemotactic capacity of pbPMNs, cells from 18 h wounded

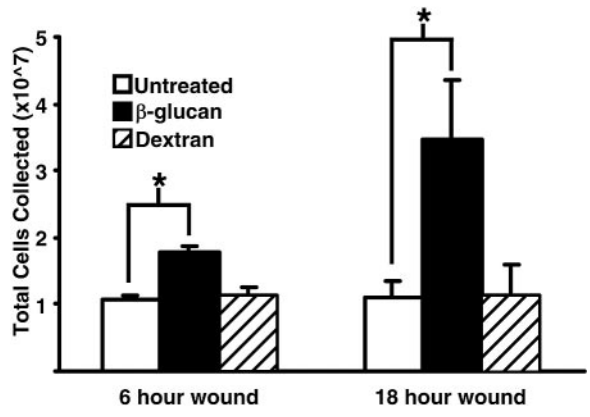


Fig. 1. Effect of β-glucan treatment on leukocyte influx into a site of sterile inflammation. Total cellular infiltrate was isolated from sponges removed at 6 and 18 h postwounding (as described in Materials and Methods) from rats treated i.v. with 2 mg/kg β-glucan, dextran (control polysaccharide), or saline. Data are expressed as the mean ± SEM. n ≥ 5 animals/group except dextran (n=3/group). *, $P < 0.05$, versus untreated rats.

animals were assayed in a modified Boyden chamber chemotaxis assay using wound fluid obtained from normal rats as a source of chemoattractants. β-Glucan treatment resulted in a significant increase in the chemotactic capacity of pbPMNs compared with PMNs obtained from untreated animals ($5.7\pm0.3\times10^5$ vs. $4.2\pm0.4\times10^5$ migrated pbPMNs from β-glucan-treated vs. -untreated animals, respectively; $P<0.05$; **Fig. 2A**). Although the increase in pbPMN chemotaxis by β-glucan treatment could be the result of changes in the expression of surface proteins necessary for emigration, flow cytometric analysis of the CXC chemokine receptor 2 (CXCR2) and CC chemokine receptor 1 (CCR1), along with CD18 and CD11b, showed no differences upon β-glucan treatment (data not shown.)

As many factors are involved in the migration of pbPMNs into a site of inflammation, experiments were performed to delineate the mechanism underlying the enhanced effect on migration by β-glucan. The chemoattractant capacity of wound fluids isolated from β-glucan-treated or -untreated animals was assayed and found to be similar for fluoresceinated pbPMNs from naïve rats ($2.3\pm0.1\times10^5$ vs. $2.2\pm0.1\times10^5$ pbPMNs migrated toward wound fluids from β-glucan-treated vs. control animals, respectively; $P>0.05$; Fig. 2B). This is supported by findings in **Table 1**, which show β-glucan treatment neither induces nor alters the cytokine/chemokine content of sera or wound fluids obtained from animals 18 h after PVA sponge implantation.

TABLE 1. Cytokine and Chemokine Protein Levels in Wound Fluid and Serum from β-Glucan-Treated and -Untreated, 18 h Wounded Rats

	Wound fluid[1]		Serum	
	Untreated	β-glucan	Untreated	β-glucan
IFN-γ[2]	188 ± 31[3]	163 ± 35	27 ± 24	n/d[4]
IL-1β	5854 ± 142	5547 ± 612	53 ± 9	69 ± 44
IL-6	4038 ± 793	3230 ± 837	165 ± 147	n/d
IL-10	252 ± 68	203 ± 55	8 ± 7	6 ± 5
TNF-α	98 ± 5	91 ± 5	69 ± 39	43 ± 20
CINC	261 ± 2	296 ± 243	131 ± 74	88 ± 32
MIP-1α	108 ± 48	81 ± 39	4 ± 7	n/d
MIP-2	235 ± 260	85 ± 34	n/d	n/d
MCP-1	297 ± 72	164 ± 141	2 ± 0.9	1 ± 0.5

[1] Wound fluid and serum were isolated from 18 h wounded rats ($n=3$ per group), treated or not with β-glucan. Aliquots were frozen and analyzed by Pierce Biotechnology Searchlight protein array enzyme-linked immunosorbent assay (ELISA) testing service. [2] All values are shown as pg/ml [except monocyte chemoattractant protein-1 (MCP-1), ng/ml]. [3] Data are means ± SEM. Statistical differences were not found among groups. [4] n/d = Not detected. IFN-γ, Interferon-γ; CINC, cytokine-induced neutrophil chemoattractant; MIP-1α, macrophage-inflammatory protein-1α.

β-Glucan effect on neutrophil:endothelial interactions

The extravasation of pb neutrophils into an inflammatory site requires adhesion to ECs and increased vascular permeability. Experiments were performed to determine whether the increase in pbPMN extravasation following β-glucan treatment included alterations in these leukocyte:EC interactions. ECIS was used to determine whether β-glucan affected neutrophil-induced loss of endothelial barrier function. pb Neutrophils were isolated from rats, treated or not with β-glucan. Cells were added to TNF-α-activated RPAECs, and the change in resistance caused by pbPMN-induced endothelial retraction was measured continuously for 3 h. Results show no difference in the change in resistance between treatment groups (**Fig. 3A**). Furthermore, results in Figure 3B show no differences in the adhesion of pbPMNs collected from β-glucan-treated compared with -untreated animals on TNF-α-activated or resting endothelial monolayers.

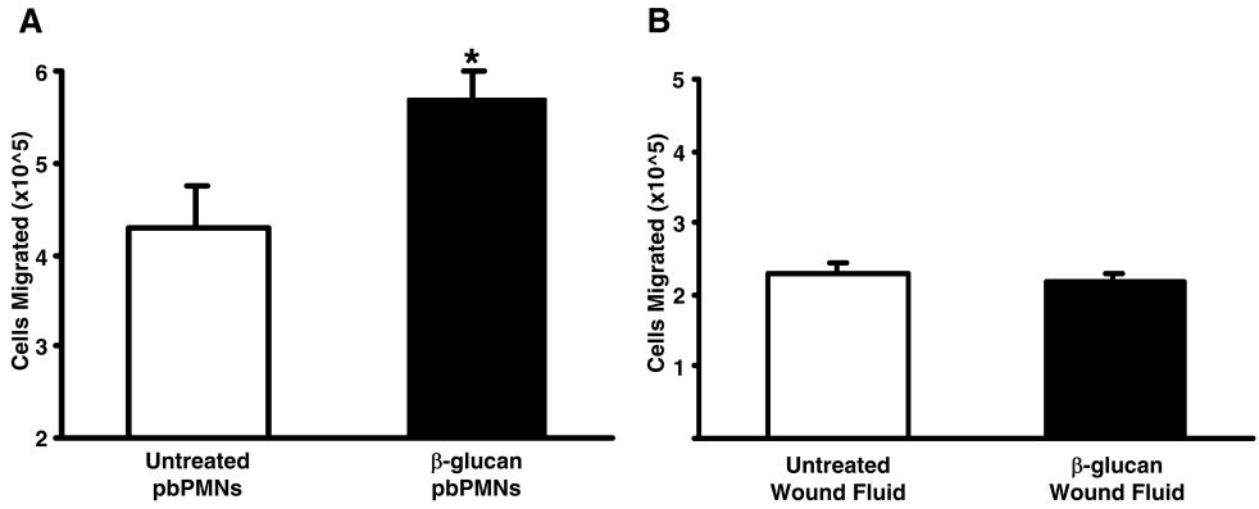


Fig. 2. Effect of β-glucan treatment on wound fluid chemotactic capacity and pbPMN chemotaxis. (A) pbPMNs from β-glucan-treated (2 mg/kg) and -untreated rats, which were wounded for 18 h, were allowed to migrate toward 18 h wound fluid. Cell migration was measured by quantitative fluorescence against a standard curve. Data expressed as the mean ± SEM. n = 5/group. *, $P < 0.05$, versus untreated pbPMNs. (B) Purified pbPMNs from naïve rats were isolated by magnetic bead separation, fluoresceinated, and allowed to migrate for 30 min toward 18 h wound fluid isolated from β-glucan-treated or -untreated animals. Cell migration was measured by quantitative fluorescence using a standard curve. Data are expressed as mean ± SEM. n ≥ 5/group. $P > 0.05$ β-glucan-treated versus -untreated wound fluid.

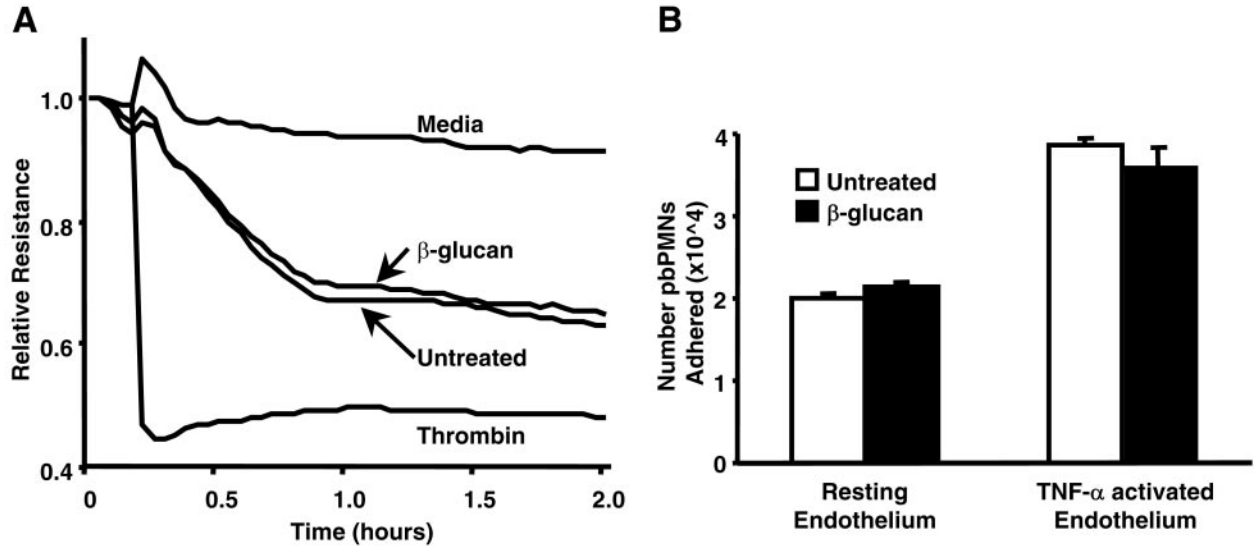


Fig. 3. The effect of β-glucan treatment on PMN:endothelial interactions. (A) Barrier function of TNF-α-activated endothelium in the presence of pbPMN from β-glucan-treated and -untreated animals. Representative tracing of electrical resistance across RPAEC monolayers measured in the absence (Media) or presence of isolated pbPMNs (β-glucan-treated or -untreated, as indicated by arrows). RPAEC monolayers were incubated with TNF-α (20 ng/ml) for 6 h at 37°C. Neutrophils from each treatment group were added to the TNF-α-activated RPAEC, and transendothelial resistance was measured for 120 min. Thrombin (0.8 U/ml) served as positive control for loss of barrier function. Tracing is representative of three independent experiments. (B) Adhesion of pbPMNs to an endothelial monolayer. RPAEC monolayers were grown to confluence and activated, or not, with TNF-α. Fluoresceinated neutrophils isolated from the pb of 18 h wounded β-glucan-treated or -untreated rats were then added, and adhesion was determined after 45 min incubation at 37°C. Data expressed as the mean ± SEM from two independent experiments (n=3; $P>0.05$).

p38 MAPK regulates the β-glucan-induced increase in pbPMN extravasation

As the p38 MAPK signaling pathway has been shown to regulate a number of neutrophil functions mediating the response to inflammatory stimuli [19], we tested the hypothesis that the increased pbPMN influx resulting from β-glucan treatment required p38 MAPK. p38 MAPK activity was inhibited using SB203580 hydrochloride at doses previously shown to have an optimal effect in vivo [20]. SB203580 blocked the increase in the number of leukocytes migrating into 6 h wounds by β-glucan treatment ($1.8\pm0.1\times10^7$ vs. $0.7\pm0.2\times10^7$, β-glucan/no inhibitor vs. β-glucan/SB203580; $P<0.01$) but had no effect on the number of cells in wounds as compared with untreated animals ($0.9\pm0.1\times10^7$ vs. $1.1\pm0.1\times10^7$, untreated/no inhibitor vs. untreated/SB203580; $P>0.05$; **Fig. 4**). Total pb leukocyte counts were not affected by treatment with β-glucan or SB203580 (not shown).

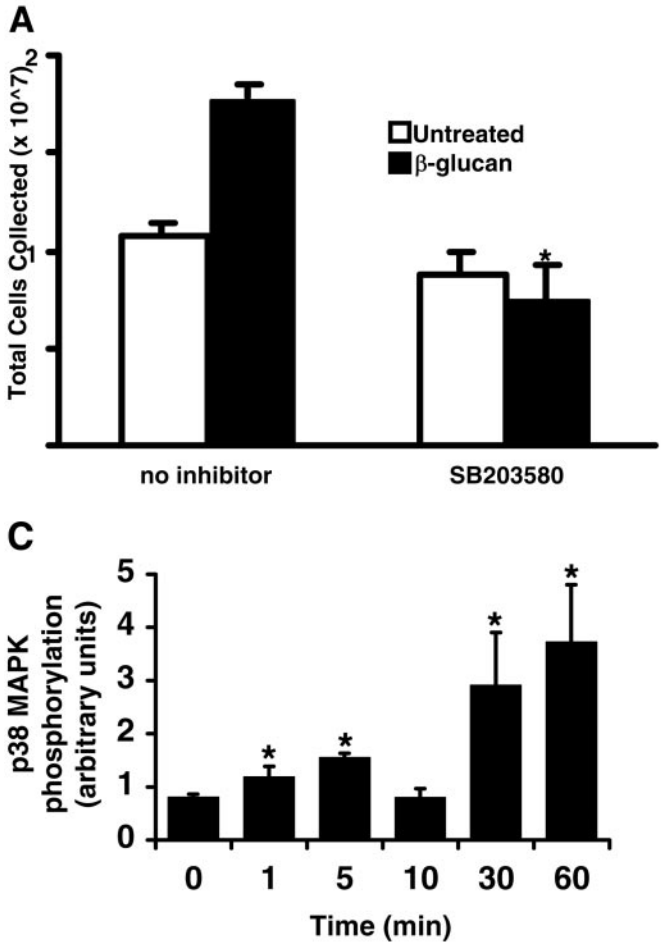
To provide direct evidence that soluble β-glucan can induce phosphorylation of p38 MAPK, freshly isolated pb neutrophils were treated for 1 h in vitro and analyzed at times indicated in Figure 4, B and C. The time course showed reproducible, biphasic p38 MAPK phosphorylation with responses over baseline at 5 min (1.9±0.1-fold increase relative to untreated, $P<0.05$) and at 60 min (4.6±1.1-fold increase relative to untreated, $P<0.05$).

β-Glucan treatment increases cellular respiratory burst activity

Having shown a potentiating effect of β-glucan on pbPMN chemotaxis, experiments were performed to determine whether other functions of inflammatory cells were altered by treatment. **Figure 5** shows the receptor-mediated O_2^- release from pbPMNs and wound cells obtained from 18 h wounded animals and plated onto adherent immune complexes. β-Glucan treatment resulted in a 40% increase in the release of O_2^- from pbPMNs by 20 min as compared with cells obtained from untreated animals. Also, a twofold increase in respiratory burst by 30 min was shown in wound cells from β-glucan-treated animals as compared with untreated animals.

β-Glucan increases leukocyte infiltration in a bacterial model of inflammation

To determine whether increased neutrophil migration occurs at a site of infection, we used a rodent model of acute pneumonia. β-Glucan was injected i.p. 18 h before and i.v. just prior to intratracheal instillation of bacteria into the rat lung. At 6 h, the lungs were lavaged, and cell number and differentials were determined. The results in **Figure 6A** show an increase in the number lavaged neutrophils from β-glucan-treated animals ($9.2\pm1.0\times10^7$ vs. $3.4\pm0.2\times10^7$ neutrophils from β-glucan-treated vs. -untreated animals, respectively; $P<0.01$), together with a significant decrease in lavaged macrophages compared with untreated controls at 6 h (2.2±0.8 vs. $5.2\pm0.3\times10^7$ macrophages from β-glucan-treated vs. -untreated animals, respectively; $P<0.05$). Furthermore, the increase in neutrophils in β-glucan-treated rats correlated with a 60% decrease in live bacteria isolated from the lavage fluid compared with untreated rats ($1.5\pm0.4\times10^9$ vs. $4.1\pm0.9\times10^9$ CFU, β-glucan-treated vs. control, respectively; $P<0.01$; Fig. 6B).



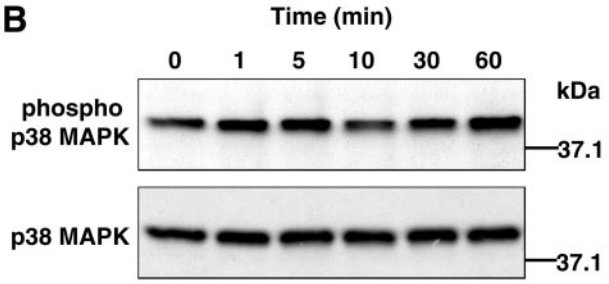


Fig. 4. Role of leukocyte p38 MAPK in response to β-glucan treatment. (A) Effect of inhibition of p38 MAPK activity on leukocyte influx into a site of sterile inflammation. Total cellular infiltrate was isolated from sponges recovered at 6 h postwounding from rats pretreated, or not, with SB203580 (20 mg/kg, i.p.) 30 min prior to wounding and β-glucan administration. Data expressed as the mean ± SEM. n ≥ 5 animals/group. *, $P < 0.01$ versus β-glucan-treated without p38 MAPK inhibitor. (B) Time course of p38 MAPK phosphorylation of freshly isolated pb neutrophils in response to soluble β-glucan. Whole neutrophil lysates were assayed for the presence of phospho-p38 MAPK (upper) and total p38 MAPK (lower). Western blots shown as a representative of three independent experiments. (C) Densitometric analysis of the ratio of phosphorylated p38 MAPK:total p38 MAPK. Data expressed as the mean ± SEM. n = 3/group; *, $P < 0.05$, versus time zero.

DISCUSSION

Previous in vitro studies from our laboratory showed effects of β-glucan on human PMNs consistent with pro- and anti-inflammatory effects. Current work assayed the effect of β-glucan on the early neutrophil-predominant phase of the inflammatory host response. Systemic administration of β-glucan increased the total cellular content of wound PVA sponges at 6 and 18 h after implantation in support of a proinflammatory effect of β-glucan. In addition, pbPMNs obtained from β-glucan-treated animals showed a heightened respiratory burst and a reduced bacterial load in a pulmonary model of infection. Findings also confirmed and extended previous reports demonstrating the unique aspect of β-glucan, which permits enhancement of PMN functions without inducing excess cytokine/chemokine production. It is this desirable property of β-glucan that allowed for its evaluation in clinical trials, where it was well-tolerated in phase III studies at doses equivalent to those used in this report and underscores its therapeutic potential as an immunomodulator [10].

The mechanism by which β-glucan increased the number of neutrophils entering the site of inflammation could not be simply explained by a corresponding increase in the number of cells present in the circulation. Systemic administration of β-glucan at the dose used in this study has been previously reported not to affect circulating leukocyte counts [6] and was confirmed herein. Therefore, the increase in neutrophil accu-

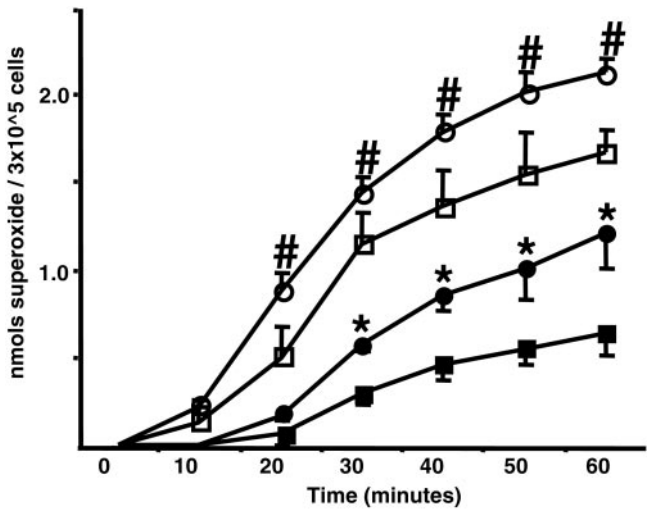


Fig. 5. β-Glucan treatment enhanced respiratory burst of isolated wound cells and pbPMNs. Wound cells and purified pbPMNs were isolated from 18 h wounded rats and placed in wells coated with BSA:anti-BSA immune complexes. The resulting O_2^- release was measured every 10 min for 1 h by cytochrome c reduction. β-Glucan-treated PMNs (○); untreated PMNs (□); β-glucan-treated wound cells (●); untreated wound cells (■). Data shown as the mean ± SD from a representative experiment of three identical experiments with n = 3/group. *, $P < 0.05$, glucan-treated versus -untreated wound cells; #, $P < 0.05$, glucan-treated versus -untreated pbPMNs.

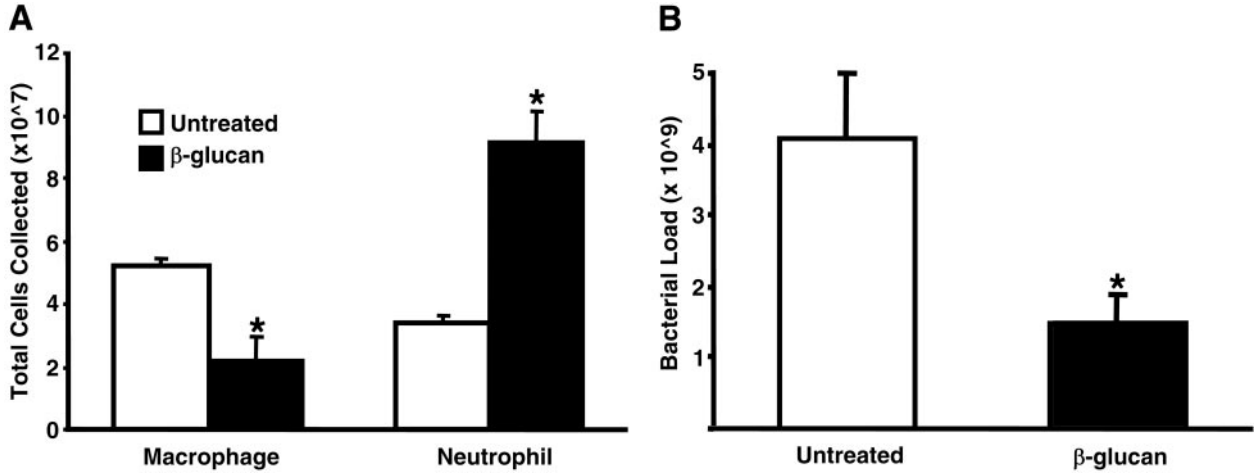


Fig. 6. Effect of β-glucan treatment on leukocyte migration and bacterial killing in a 6 h *E. coli* pneumonia. (A) Leukocytes isolated from the BAL fluid of pneumonic rats treated, or not, with β-glucan. Data expressed as the mean ± SEM. n = 3/group. *, $P < 0.01$, β-glucan-treated versus -untreated. (B) Live bacteria isolated from the lavage fluid of pneumonic rats treated, or not, with β-glucan, and β-glucan treatment resulted in decreased live bacteria isolated from lavage fluid. Data expressed as the mean ± SEM. n = 9/group. *, $P < 0.01$, β-glucan treated versus -untreated.

mulation into a site of injury was consistent with a gain-of-function in chemotactic capacity of neutrophils as seen in vitro [12] and confirmed ex vivo (Fig. 2A).

As neutrophil extravasation entails a step-wise series of events, studies were undertaken to determine the specific points within the process that are affected by β-glucan. First, an increase in cytokines or chemokines released by cells in the wound site in β-glucan-treated animals could have contributed to the increased accumulation of leukocytes in the wound site, as β-glucan itself has no intrinsic chemotactic capacity (unpublished observation). However, no difference in the chemotactic capacity of wound fluids isolated from β-glucan-treated or -untreated animals was seen in assays using pbPMNs from naïve animals in a modified Boyden chamber (Fig. 2B). Further, levels of individual chemokines and cytokines present in the wound fluids and the sera of β-glucan-treated and -untreated rats were measured and found to be the same (Table 1). This is in agreement with previous studies showing that β-glucan treatment of murine or human PMNs ex vivo does not elicit release of IL-1β, TNF-α, IL-6, IL-8, keratinocyte-derived chemokine (KC), or MIP-2 [6, 9]. The relevance of these experiments was extended in the current report by showing that β-glucan treatment in vivo did not cause increased cytokine release in wounded animals, even while showing a promotional effect on neutrophil extravasation. Moreover, β-glucan did not alter the surface expression of CXCR2 or CCR1, which are receptors for the prevailing chemokines expressed during early inflammation on pbPMNs [21–23]. Taken together, these findings suggest that the increased response of pbPMNs to a site of sterile inflammation induced by β-glucan is not significantly mediated through altered modulation of chemoattractants or expression of cell-surface chemokine receptors (CRs).

Neutrophil extravasation requires adhesive interactions with the vascular endothelium, which often result in loss of endothelial barrier function. In fact, vascular leakage in inflammation is dependent on neutrophils [24]. Recent in vitro findings from our laboratory showed that the addition of β-glucan maintained the barrier function of endothelial monolayers during challenge with activated neutrophils and did so without altering the strength of adhesion between the neutrophils and the activated endothelium [14]. β-Glucan was also shown to reduce neutrophil transmigration across an endothelial monolayer. Neutrophils isolated from untreated or β-glucan-treated rats were not different in adhesion to ECs or in their ability to induce loss of endothelial barrier function. Moreover, the increased migration of PMNs into inflammatory sites following β-glucan treatment shows that the reduced transendothelial migration observed in vitro may not be rate-limiting in vivo. Taken together, the current study suggests that β-glucan exerts a direct effect on the chemotactic capacity of the pbPMN.

We have not yet determined the substrate(s) for β-glucan-induced p38 MAPK, which leads to increased PMN migration. Molecules downstream of p38 MAPK, such as heat shock protein 27, are known to regulate cell motility and are therefore logical candidates. In addition, leukocyte-specific protein (LSP1) is an F-actin, bundling protein present in neutrophils and ECs and is a major substrate of MAPK activating protein (MAPKAP) kinase 2, which itself is a kinase activated by p38 MAPK [25]. Although LSP1 has been thought to restrict cytoskeletal rearrangements, thereby limiting neutrophil chemotaxis, in a recent landmark study, Kubes and colleagues have shown that neutrophil transendothelial migration is regulated by endothelial, not neutrophil, LSP1 [26]. ECs from LSP1−/− mice permitted greater PMN transmigration than ECs from wild-type animals. Vascular ECs have been shown to express receptors for β-glucan [27]; however, prior work from this laboratory showed that β-glucan had no direct effect on the barrier function of ECs [14]. Therefore, although LSP1 is a major target of MAPKAP kinase 2 and is sensitive to inhibition of p38 MAPK, it is unlikely to mediate the augmented extravasation of PMNs into PVA sponges by β-glucan.

Using the model of sterile inflammation provided by PVA sponge implantation, we show that normal PMN extravasation is independent of p38 MAPK but that β-glucan-induced en-

hancement of extravasation requires active p38 MAPK. The reduction in the number of inflammatory cells accumulating in PVA sponges also confirms that the SB203580 was pharmacologically active under the conditions used in our studies. The p38 MAPK-independent nature of leukocyte entry into sterile PVA sponges in the absence of β-glucan contrasts with the finding of others, where signaling through the p38 MAPK pathway was shown to regulate the leukocytic inflammatory response in vivo. For example, PMN accumulation in the lung in response to intratracheal instillation of lipopolysaccharide (LPS) and KC is p38 MAPK-dependent [28, 29]. Furthermore, a direct, functional role of p38 MAPK during neutrophil chemotaxis toward KC was determined using intravital microscopy [20]. PVA sponge implantation, like most injuries, elicits multiple chemoattractants and stands in contrast to models using a singular chemokine as mentioned above. The availability of multiple chemoattractants in a sterile wound microenvironment may serve to circumvent the p38 MAPK dependence of migration to a single chemokine.

A role for phosphatidylinositol-3 kinase (PI-3K) in regulating directional cell movement has been shown in mice lacking p110γ in which PMNs were defective in fMLP-induced chemotaxis but showed no impairment in random motility [30]. More recently, specific inhibition of the PI-3K δ isoform was shown to regulate the directed but not random motility of human PMNs in vitro as well as to reduce accumulation of PMNs into inflamed tissues in vivo [31, 32]. Whether β-glucan priming affects the PI-3K pathway in addition to p38 MAPK is currently being investigated.

Macrophages isolated from sterile PVA sponges as early as 3 days following implantation are deficient in their capacity to generate reactive oxygen intermediates [17]. Whether the wound microenvironment is similarly nonsupportive of the respiratory burst during the early, neutrophil-predominant phase of the host response had not been examined previously. Moreover, whether the β-glucan-primed state of the pb leukocyte is maintained following extravasation into an otherwise nonsupportive microenvironment could not be presumed. We show here a diminution of the respiratory burst elicited from extravasated as compared with circulating PMNs in normal and β-glucan-treated animals. In untreated animals, this reduction did not reach the absolute abrogation reported for wound macrophages; however, the data suggest that the sterile wound microenvironment imparts a regulatory effect on the respiratory burst activity on inflammatory neutrophils. PMNs isolated from the sponges of β-glucan-treated animals did show a significant increase in O_2^- production as compared with untreated animals such that β-glucan priming is maintained after tissue entry.

The therapeutic potential of β-glucan for treating noninfectious damage in organs/tissues such as in arthritis, chronic obstructive pulmonary disease, traumatic injury, or multiorgan failure is not known. The present study showed a heightened level of O_2^- production in wound-derived PMNs from β-glucan-treated rats but whether this is sufficient to exacerbate tissue damage or whether β-glucan has a reparative effect on tissue injury requires further study.

The use of a local model of bacterial challenge extends the therapeutic relevance of β-glucan treatment by demonstrating that increased extravasation is accompanied by an enhanced antimicrobial response. Neutrophil extravasation into the lung requires CD11/CD18 adhesion molecules in response to *E. coli*, *E. coli* LPS, *Pseudomonas aeruginosa*, IgG immune complexes, and PMA [33]. In contrast, *Streptococcus pneumonia*, Group B *Streptococci*, *Staphylococcus aureus*, and C5a elicit neutrophil responses that do not require CD11/CD18. The relevance of this selective mechanism of PMN migration to the current study lies in the demonstrated role of CR3 as a receptor for β-glucan on neutrophils, in addition to the role of CR3 as an adhesion molecule. Therefore, whether β-glucan is altering only CR3-dependent antimicrobial responses is possible but awaits confirmation in future studies.

In summary, this report outlines the mechanism by which β-glucan treatment results in an increase in the migratory potential of neutrophils in in vivo models of inflammation. Through recognition of β-glucan in the circulation, the neutrophil undergoes functional augmentation, resulting in the acquisition of increased interstitial migratory potential by a p38 MAPK-dependent mechanism, which is inhibitable by SB203580. This neutrophil influx increase does not correlate with an increase in chemokine content in the inflammatory site and is shown here to be beneficial to host defense in a model of local infection.

ACKNOWLEDGMENTS

This work was supported by the National Institutes of Health (Grants GM66194 to J. S. R. and GM42859 to J. E. A.), a Grant in Assistance to Areas of National Need from the U.S. Department of Education, P200A030100 (B. W. L.), and allocations to the Department of Surgery by Rhode Island Hospital. The authors thank Xian O'Brien for critical reading of the manuscript.

REFERENCES

1. Ley, K. (2002) Integration of inflammatory signals by rolling neutrophils. *Immunol. Rev.* **186,** 8–18.
2. Gautam, N., Herwald, H., Hedqvist, P., Lindbom, L. (2000) Signaling via β(2) integrins triggers neutrophil-dependent alteration in endothelial barrier function. *J. Exp. Med.* **191,** 1829–1839.
3. Gautam, N., Olofsson, A. M., Herwald, H., Iversen, L. F., Lundgren-Akerlund, E., Hedqvist, P., Arfors, K. E., Flodgaard, H., Lindbom, L. (2001) Heparin-binding protein (HBP/CAP37): a missing link in neutrophil-evoked alteration of vascular permeability. *Nat. Med.* **7,** 1123–1127.
4. Lindbom, L., Werr, J. (2002) Integrin-dependent neutrophil migration in extravascular tissue. *Semin. Immunol.* **14,** 115–121.
5. Brown, G. D., Gordon, S. (2003) Fungal β-glucans and mammalian immunity. *Immunity* **19,** 311–315.
6. Patchen, M. L., Liang, J., Vaudrain, T., Martin, T., Melican, D., Zhong, S., Stewart, M., Quesenberry, P. J. (1998) Mobilization of peripheral blood progenitor cells by betafectin PGG-glucan alone and in combination with granulocyte colony-stimulating factor. *Stem Cells* **16,** 208–217.
7. Adams, D. S., Nathans, R., Pero, S. C., Sen, A., Wakshull, E. (2000) Activation of a rel-A/CEBP-β-related transcription factor heteromer by PGG-glucan in a murine monocytic cell line. *J. Cell. Biochem.* **77,** 221–233.
8. Adams, D. S., Pero, S. C., Petro, J. B., Nathans, R., Mackin, W. M., Wakshull, E. (1997) PGG-glucan activates NF-κB-like and NF-IL-6-like transcription factor complexes in a murine monocytic cell line. *J. Leukoc. Biol.* **62,** 865–873.
9. Wakshull, E., Brunke-Reese, D., Lindermuth, J., Fisette, L., Nathans, R. S., Crowley, J. J., Tufts, J. C., Zimmerman, J., Mackin, W., Adams,

D. S. (1999) PGG-glucan, a soluble β-(1,3)-glucan, enhances the oxidative burst response, microbicidal activity, and activates an NF-κ B-like factor in human PMN: evidence for a glycosphingolipid β-(1,3)-glucan receptor. *Immunopharmacology* **41,** 89–107.
10. Dellinger, E. P., Babineau, T. J., Bleicher, P., Kaiser, A. B., Seibert, G. B., Postier, R. G., Vogel, S. B., Norman, J., Kaufman, D., Galandiuk, S., Condon, R. E. (1999) Effect of PGG-glucan on the rate of serious postoperative infection or death observed after high-risk gastrointestinal operations. Betafectin Gastrointestinal Study Group. *Arch. Surg.* **134,** 977–983.
11. Babineau, T. J., Hackford, A., Kenler, A., Bistrian, B., Forse, R. A., Fairchild, P. G., Heard, S., Keroack, M., Caushaj, P., Benotti, P. (1994) A phase II multicenter, double-blind, randomized, placebo-controlled study of three dosages of an immunomodulator (PGG-glucan) in high-risk surgical patients. *Arch. Surg.* **129,** 1204–1210.
12. Harler, M. B., Wakshull, E., Filardo, E. J., Albina, J. E., Reichner, J. S. (1999) Promotion of neutrophil chemotaxis through differential regulation of β 1 and β 2 integrins. *J. Immunol.* **162,** 6792–6799.
13. Tsikitis, V. L., Albina, J. E., Reichner, J. S. (2004) β-Glucan affects leukocyte navigation in a complex chemotactic gradient. *Surgery* **136,** 384–389.
14. Tsikitis, V. L., Morin, N. A., Harrington, E. O., Albina, J. E., Reichner, J. S. (2004) The lectin-like domain of complement receptor 3 protects endothelial barrier function from activated neutrophils. *J. Immunol.* **173,** 1284–1291.
15. Meszaros, A. J., Reichner, J. S., Albina, J. E. (2000) Macrophage-induced neutrophil apoptosis. *J. Immunol.* **165,** 435–441.
16. Sekiya, S., Gotoh, S., Yamashita, T., Watanabe, T., Saitoh, S., Sendo, F. (1989) Selective depletion of rat neutrophils by in vivo administration of a monoclonal antibody. *J. Leukoc. Biol.* **46,** 96–102.
17. Nessel, C. C., Henry Jr., W. L., Mastrofrancesco, B., Reichner, J. S., Albina, J. E. (1999) Vestigial respiratory burst activity in wound macrophages. *Am. J. Physiol.* **276,** R1587–R1594.
18. Wegener, J., Keese, C. R., Giaever, I. (2000) Electric cell-substrate impedance sensing (ECIS) as a noninvasive means to monitor the kinetics of cell spreading to artificial surfaces. *Exp. Cell Res.* **259,** 158–166.
19. Herlaar, E., Brown, Z. (1999) p38 MAPK signaling cascades in inflammatory disease. *Mol. Med. Today* **5,** 439–447.
20. Cara, D. C., Kaur, J., Forster, M., McCafferty, D. M., Kubes, P. (2001) Role of p38 mitogen-activated protein kinase in chemokine-induced emigration and chemotaxis in vivo. *J. Immunol.* **167,** 6552–6558.
21. Dipietro, L. A., Reintjes, M. G., Low, Q. E., Levi, B., Gamelli, R. L. (2001) Modulation of macrophage recruitment into wounds by monocyte chemoattractant protein-1. *Wound Repair Regen.* **9,** 28–33.
22. Devalaraja, R. M., Nanney, L. B., Du, J., Qian, Q., Yu, Y., Devalaraja, M. N., Richmond, A. (2000) Delayed wound healing in CXCR2 knockout mice. *J. Invest. Dermatol.* **115,** 234–244.
23. Engelhardt, E., Toksoy, A., Goebeler, M., Debus, S., Brocker, E. B., Gillitzer, R. (1998) Chemokines IL-8, GROα, MCP-1, IP-10, and Mig are sequentially and differentially expressed during phase-specific infiltration of leukocyte subsets in human wound healing. *Am. J. Pathol.* **153,** 1849–1860.
24. Edens, H. A., Parkos, C. A. (2003) Neutrophil transendothelial migration and alteration in vascular permeability: focus on neutrophil-derived azurocidin. *Curr. Opin. Hematol.* **10,** 25–30.
25. Jongstra, J., Tidmarsh, G. F., Jongstra-Bilen, J., Davis, M. M. (1988) A new lymphocyte-specific gene which encodes a putative Ca2+-binding protein is not expressed in transformed T lymphocyte lines. *J. Immunol.* **141,** 3999–4004.
26. Liu, L., Cara, D. C., Kaur, J., Raharjo, E., Mullaly, S. C., Jongstra-Bilen, J., Jongstra, J., Kubes, P. (2005) LSP1 is an endothelial gatekeeper of leukocyte transendothelial migration. *J. Exp. Med.* **201,** 409–418.
27. Lowe, E. P., Wei, D., Rice, P. J., Li, C., Kalbfleisch, J., Browder, I. W., Williams, D. L. (2002) Human vascular endothelial cells express pattern recognition receptors for fungal glucans which stimulates nuclear factor κB activation and interleukin 8 production. Winner of the Best Paper award from the Gold Medal Forum. *Am. Surg.* **68,** 508–517.
28. Nick, J. A., Young, S. K., Brown, K. K., Avdi, N. J., Arndt, P. G., Suratt, B. T., Janes, M. S., Henson, P. M., Worthen, G. S. (2000) Role of p38 mitogen-activated protein kinase in a murine model of pulmonary inflammation. *J. Immunol.* **164,** 2151–2159.
29. Nick, J. A., Young, S. K., Arndt, P. G., Lieber, J. G., Suratt, B. T., Poch, K. R., Avdi, N. J., Malcolm, K. C., Taube, C., Henson, P. M., Worthen, G. S. (2002) Selective suppression of neutrophil accumulation in ongoing pulmonary inflammation by systemic inhibition of p38 mitogen-activated protein kinase. *J. Immunol.* **169,** 5260–5269.
30. Hannigan, M. O., Huang, C. K., Wu, D. Q. (2004) Roles of PI3K in neutrophil function. *Curr. Top. Microbiol. Immunol.* **282,** 165–175.
31. Puri, K. D., Doggett, T. A., Douangpanya, J., Hou, Y., Tino, W. T., Wilson, T., Graf, T., Clayton, E., Turner, M., Hayflick, J. S., Diacovo, T. G. (2004) Mechanisms and implications of phosphoinositide 3-kinase δ in promoting neutrophil trafficking into inflamed tissue. *Blood* **103,** 3448–3456.
32. Sadhu, C., Masinovsky, B., Dick, K., Sowell, C. G., Staunton, D. E. (2003) Essential role of phosphoinositide 3-kinase δ in neutrophil directional movement. *J. Immunol.* **170,** 2647–2654.
33. Doerschuk, C. M., Tasaka, S., Wang, Q. (2000) CD11/CD18-dependent and -independent neutrophil emigration in the lungs: how do neutrophils know which route to take? *Am. J. Respir. Cell Mol. Biol.* **23,** 133–136.