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Differential regulation of oxidative burst by distinct β-glucan-binding receptors and signaling pathways in human peripheral blood mononuclear cells

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β-Glucans possess broad immunomodulatory properties, including activation of innate immune functions such as oxidative burst activity. The differential roles of complement receptor type 3 (CR3) and Dectin-1, the known β-glucan receptors, and their associated signaling pathways in the generation of oxidative burst induced by different physical forms of *Saccharomyces cerevisiae*-derived β-glucan were examined in human peripheral blood mononuclear cells (PBMC). In this study whole glucan particle (WGP) or immobilized soluble β-glucan (ISG) was used to represent the phagocytizable or the nonphagocytizable form of a fungus, respectively. Oxidative burst as measured by the formation of superoxide (SO) was detected in PBMC in response to WGP and ISG. SO induction with WGP was concluded to be Dectin-1-mediated and required Src family kinases, phosphatidylinositol-3 kinase and protein kinase B/Akt. In contrast, the SO induction generated by ISG was CR3-mediated and required focal adhesion kinase, spleen tyrosine kinase, phosphatidylinositol-3 kinase, Akt, p38 mitogen activated protein kinase, phospholipase C and protein kinase C. The study results support the hypothesis that human PBMC, specifically monocytes, utilize distinct receptors and overlapping, but distinct, signaling pathways for the oxidative burst in response to challenge by different physical forms of β-glucan.

Keywords: CR3 / Dectin-1 / human monocytes / oxidative burst / β-glucan

Introduction

β-Glucans are conserved microbial structures found in unicellular and multicellular pathogens, but not found in mammalian cells, and thus are considered pathogen-associated molecular patterns (PAMP) (Mogensen 2009). They possess broad immunomodulatory properties, including activation of innate immune functions such as oxidative burst activity. Fungal β-glucans are a diverse class of long-chain glucose polymers consisting of a backbone of glucose residues linked by β-(1,3)-glycosidic bonds with attached side-chain glucose residues joined to the backbone chain by β-(1,6) linkages (Thompson et al. 2010). A number of studies indicate that β-glucan is a key recognition element for phagocytes mediating immune responses to fungal pathogens, such as *Pneumocystis carinii* and *Candida albicans* (Brown et al. 2003; Steele et al. 2003; Gantner et al. 2005; Willment et al. 2005).

In general, phagocytes engulf unicellular pathogens and destroy them within phagolysosomes by undergoing an oxidative burst. In an oxidative burst response, first the rate of oxygen consumption increases extensively. Subsequently, oxygen is reduced to superoxide (SO) anion and several other reactive oxygen intermediates by a plasma-membrane-bound nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase enzyme. Oxidative burst also occurs when phagocytes attempt to engulf multicellular pathogens and fail. This results in the release of oxidative products into the environment, a process referred to as "frustrated phagocytosis" (Henson 1971; Lavigne et al. 2006). Previous studies have demonstrated that purified β-glucan can induce phagocytosis-dependent or frustrated-phagocytosis-dependent oxidative burst in phagocytes from different species, including human neutrophils and rat macrophages (Michalek et al. 1998; Brown 2005; Brown 2006; Lavigne et al. 2006; Thompson et al. 2010).

Cell surface receptors on innate immune cells which recognize β-glucan, including Dectin-1 and complement receptor type 3 (CR3), have been the subject of detailed reviews (Brown 2005; Goodridge, Wolf et al. 2009; Marakalala et al. 2011; Geijtenbeek and Gringhuis 2009; Netea and Maródi 2010; Brown and Williams 2009). Dectin-1 signaling has been characterized in great molecular detail. Significant parallels have been drawn between classical immunoreceptor- and Dectin-1-mediated signaling due to the presence of the cytoplasmic immunoreceptor tyrosine-based activation motif. Signaling through Dectin-1 after β-glucan ligation has been shown to result in activation of

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Src family kinases (SFKs), spleen tyrosine kinase (Syk), phosphatidylinositol-3 kinase (PI3K), Akt, mitogen activated protein kinase (MAPK) and phospholipase C (PLC). These studies have also shown that the signaling molecules involved depend on the functional response being evaluated in a given cell type (Gantner et al. 2003; Herre et al. 2004; Rogers et al. 2005; Underhill et al. 2005; Dennehy et al. 2008; Goodridge, Shimada et al. 2009; Shah et al. 2009; Xu, Huo et al. 2009). Although CR3-mediated signaling pathways in response to various I-domain ligands have been the focus of intense investigation, β-glucan-mediated CR3-signaling pathways downstream of lectin domain ligation are less characterized. Syk, PI3K and p38 MAPK activation has been shown in human neutrophils following β-glucan ligation of CR3 (Berton and Lowell 1999, Lavigne et al. 2006; Li et al. 2006; Mócsai et al. 2006).

Previous studies elucidating relevant β-glucan receptors, signaling pathways and cellular responses have used soluble as well as particulate β-glucan preparations from various sources. Some of these β-glucans contained impurities such as mannans and mannoproteins (Goldman et al. 1994). The lack of purified and well-characterized β-glucan has contributed to the difficulties deciphering the mechanism of β-glucan-induced responses. The majority of studies of the oxidative burst response induced by β-glucans via Dectin-1 or CR3 have used zymosan or intact yeast which contain β-glucan along with impurities like mannans, chitosan and other cell wall components (Goldman et al. 1994; Thornton et al. 1996; Gantner et al. 2003; Underhill et al. 2005; Lee et al. 2007). Furthermore, few studies have evaluated the mechanism of purified β-glucan-induced oxidative burst in human peripheral blood mononuclear cells (PBMC). In the present study we tested the hypothesis that different forms of β-glucans will engage different phagocyte receptors, and consequently will activate different signaling pathways to generate oxidative burst in PBMC. We used purified β-glucan particles or soluble β-glucan presented on a surface to represent forms of fungi that are phagocytizable or that induce frustrated phagocytosis, respectively. This study demonstrates that different physical forms of β-glucan can induce oxidative burst in PBMC by utilizing different receptors and signaling molecules.

Results

Detection of oxidative burst in response to both physical forms of β-glucan

We used two different physical forms of yeast *Saccharomyces cerevisiae* β-glucan represented by whole glucan particle (WGP) and immobilized soluble β-glucan (ISG) to stimulate human PBMC and measure oxidative burst. WGP induced a dose-dependent SO production rate ranging from 2 to 15 nmol with a maximal response at concentrations >480 μg/mL (Figure 1A). For ISG-induced bursts, the range of SO production was 0.6–8 nmol, with a maximal response occurring at plate-coating concentrations >12.5 μg/mL (Figure 1B). As given in Table I, the percentage of contaminants, including mannans and proteins in the β-glucan used is extremely small. Regardless, we tested whether mannan is a likely contributor to the oxidative burst response as it is a mannose containing PAMP. Yeast mannan when tested at concentrations 3.1–12.5 μg/mL, which is ~3–10-fold

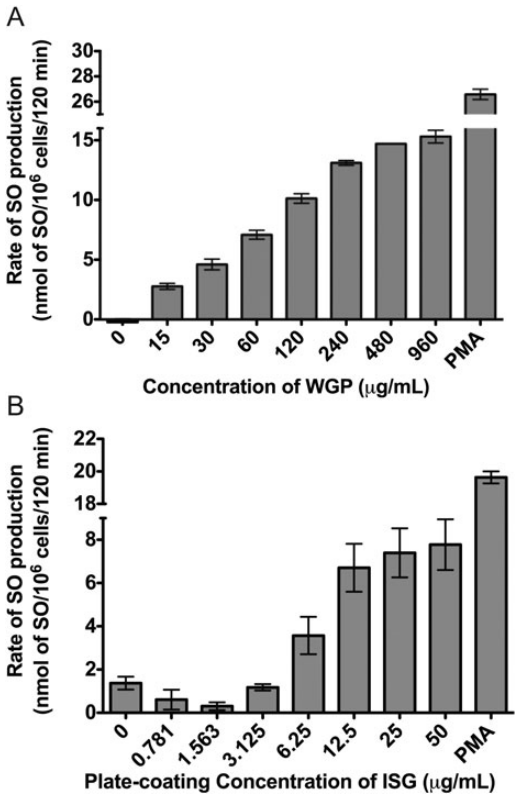

Fig. 1. Oxidative burst response of human PBMC to WGP and ISG. SO production by human PBMC exposed to increasing concentrations of WGP (**A**), or ISG (**B**) at 37°C for 120 min was measured as described in the Materials and methods section. The response of PBMC at each concentration is represented as rate of SO production (nmol of SO/10^6 cells/120 min). Values plotted in the graph are mean values ± SD (standard deviation) from triplicate wells in one experiment using one donor; these results are representative of at least two independent experiments from different donors.

Table I. Analytical characterization of a representative lot of WGP and ISG

Parameters	WGP	ISG
Purity[a]		
Residual protein	2.8%[b]	≤ 0.2% (value lower than LOQ)[c]
% Mannans (mannose)[d]	0.5%	≤ 0.6% (value lower than LOQ)
% Glycogen[e]	0.6%	≤ 5% (value lower than LOQ)
% Chitin (glucosamine)[f]	4%	0.4%
Linkage[g]		
% 1,3-linked glucose units	77.2	85.9
% 1,3-/1,6-linked glucose units	5.1	3.7
% 1,6-linked glucose units	7.1	2.0

[a]Expressed as % of total hexose.
[b]Not more than 2.8% protein as determined by multiplying 6.25 by the % N found by elemental analysis.
[c]Detection by Bradford protein assay.
[d]Hydrolysis with trifluoroacetic acid to monosaccharides followed by high-performance anion exchange chromatography with pulsed amperometric detection.
[e]Digestion by amyloglucosidase and detection of liberated glucose by an enzymatic glucose detection assay.
[f]Hydrolysis with sulfuric acid followed by reaction with acetyl acetone and Ehrlich's reagent and detection spectrophotometrically.
[g]Partially methylated alditol acetate method using gas chromatography with flame ionization detection.

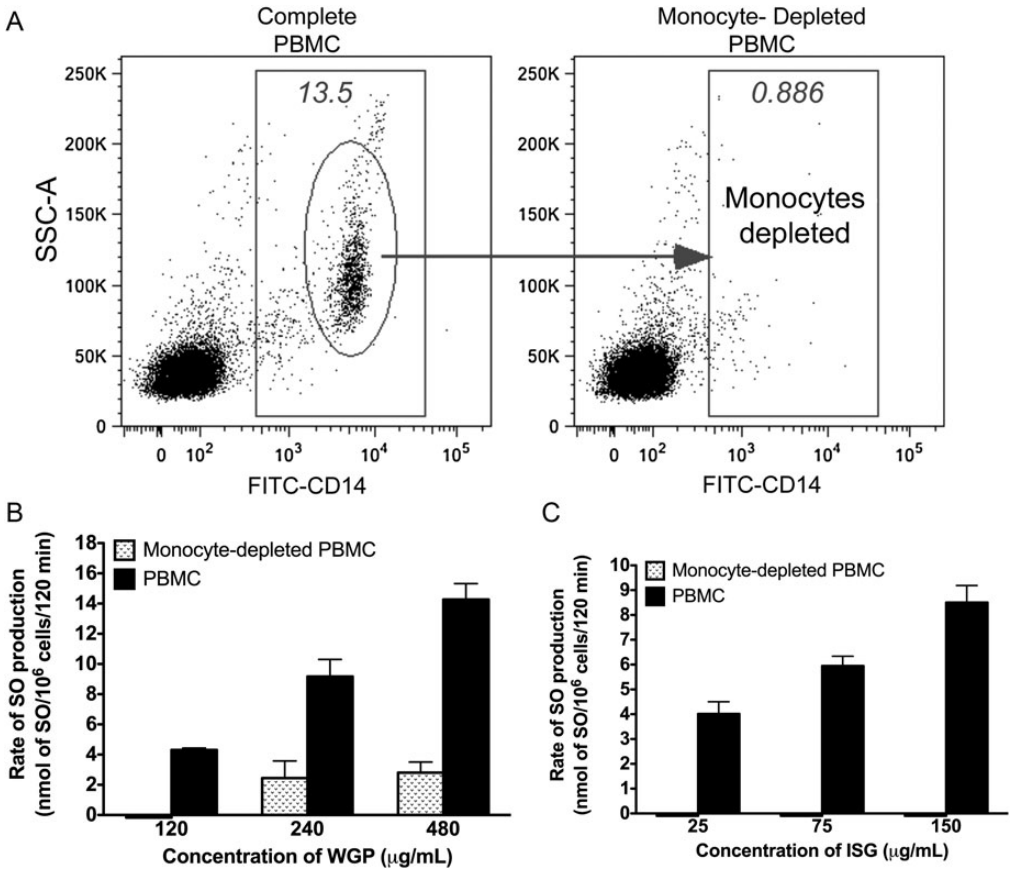

Fig. 2. Inhibition of WGP and ISG-induced oxidative burst after depletion of monocytes from human PBMC. Human PBMC were depleted of monocytes using CD14 microbeads as described in the Materials and Methods section. The cells were checked for monocyte depletion by staining before and after with CD14-specific antibody. The plot shows the side scatter area (SSC-A) versus the fluorescein isothiocyanate (FITC) staining of CD14. (**A**) Complete and monocyte-depleted PBMC were subsequently stimulated with the indicated concentrations of WGP (**B**) or ISG (**C**) at 37°C for 120 min and SO production was measured. The response of PBMC to β-glucan is represented as rate of SO production (nmol of SO/10^6 cells/120 min). As 50–80% of the oxidative burst induced by PMA was also affected by monocyte depletion, the cells were judged viable; viability was also verified by a trypan blue exclusion test (data not shown). Values plotted on the graph are mean values ± SD from duplicate wells in one experiment using one donor; these results are representative of at least two independent experiments from different donors.

higher than the allowed limit of mannan content at the highest concentration of ISG (0.6% of 150 μg/mL β-glucan is 0.9 μg/mL), failed to induce any oxidative burst response (Supplementary data, Figure S1). The difference in the production of SO induced by ISG and WGP raised the question of whether there were qualitative differences in the β-glucan-stimulated signaling pathway that depend on the physical form of the ligand being recognized by specific receptors.

Role of monocytes within the PBMC population in generating the oxidative burst

The monocyte sub-population of human PBMC has been extensively studied as the producer of reactive oxygen species (ROS) as a consequence of phagocytosis, or stimulation with a variety of agents including immune complexes, chemotactic peptides and protein kinase activators (Bonvini et al. 1984; Kharazmi et al. 1989; Trial et al. 1995). To investigate the role of monocytes in oxidatively responding to either WGP or ISG, monocytes were depleted from PBMC by using CD14 magnetic microbeads (Figure 2A) and the monocyte-depleted PBMC were subsequently assayed for an oxidative burst response. The cells were judged viable by a trypan blue exclusion test (data not shown). As shown in Figure 2B and C, monocyte removal reduced the rate of SO production in both WGP- and ISG-stimulated PBMC by 75–100%, implicating monocytes within the PBMC population as the primary cells responsible for the oxidative burst response.

Different receptors mediate the oxidative bursts induced by WGP and ISG in human PBMC

After the level of expression of Dectin-1 and CR3 on the monocytes was determined (Figure 3), the β-glucan-induced oxidative burst activity was measured with and without blocking these receptors using receptor-specific monoclonal antibodies. The Dectin-1 receptor was blocked using GE2, the monoclonal antibody that has been previously demonstrated to inhibit Dectin-1 mediated functions (Willment et al. 2005). The CR3 receptor was blocked using a combination of antibodies to both the CD11b and CD18 subunits as it was more effective in comparison with blocking the CD11b chain alone (data not shown). Moreover, even though CD11b has been shown to compose the

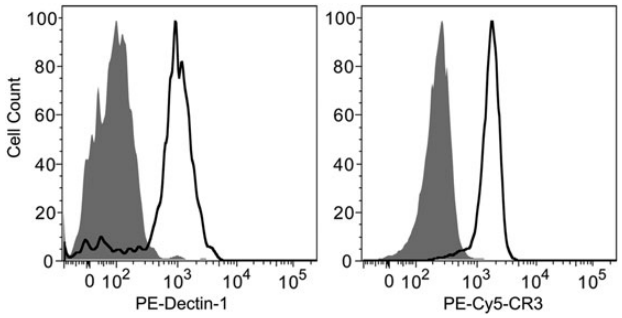

Fig. 3. Verification of Dectin-1 and CR3 expression on human monocytes from PBMC. The expression of Dectin-1 and CR3 on human monocytes was evaluated by staining for Dectin-1 using GE2 as the primary antibody followed by phycoerythrin (PE)-labeled goat anti-mouse secondary antibody and for CR3 using ICRF44-PE/Cy5 antibody. Human PBMC (1×10^6) were incubated with the staining antibodies or relevant isotype controls for 30 min on ice, followed by two washes with immunofluorescence buffer and then data acquisition by flow cytometry. The histograms show expression of Dectin-1 or CR3 (solid lines) in comparison with isotype control (shaded gray) on CD14+ monocytes in PBMC. Log scale of fluorescence intensity is shown on the *x*-axis. Data shown here are representative of two independent experiments.

ligand-binding domains, the CD18 chain has been shown to be critical to signaling events (Ross et al. 1985; Xia and Ross 1999). IB4 antibody was used to block CD18 (β-chain), while LM2/1 and VIM12 antibodies were used to block the I-domain and the lectin domain of CD11b (α-chain), respectively. PBMC pretreated with blocking antibodies or isotype controls were incubated with either WGP (240 and 960 μg/mL) or ISG (6.25 and 25 μg/mL) and production of SO was measured. Blocking the Dectin-1 receptor (Figure 4A) resulted in 40% inhibition of the WGP-induced oxidative burst, while CR3 blocking antibodies did not affect the activity (Figure 4B). In contrast to the WGP-induced activity, the ISG-induced oxidative burst was unaffected by Dectin-1 blocking antibodies (Figure 4C); however, the anti-CR3 antibodies (Figure 4D) inhibited the response by 70–80%. Treatment of the cells with the nonspecific isotype controls did not show similar inhibition of the oxidative burst activity of the cells. Similar results were also obtained in isolated monocytes enriched from the PBMC population (Supplementary data, Figure S2). These results show that Dectin-1 is involved in WGP-induced oxidative burst, while CR3 plays an important role in ISG-induced oxidative burst activity in human PBMC. After confirming that the role of CR3 and Dectin-1 in WGP- and ISG-mediated oxidative burst is the same in both PBMC and

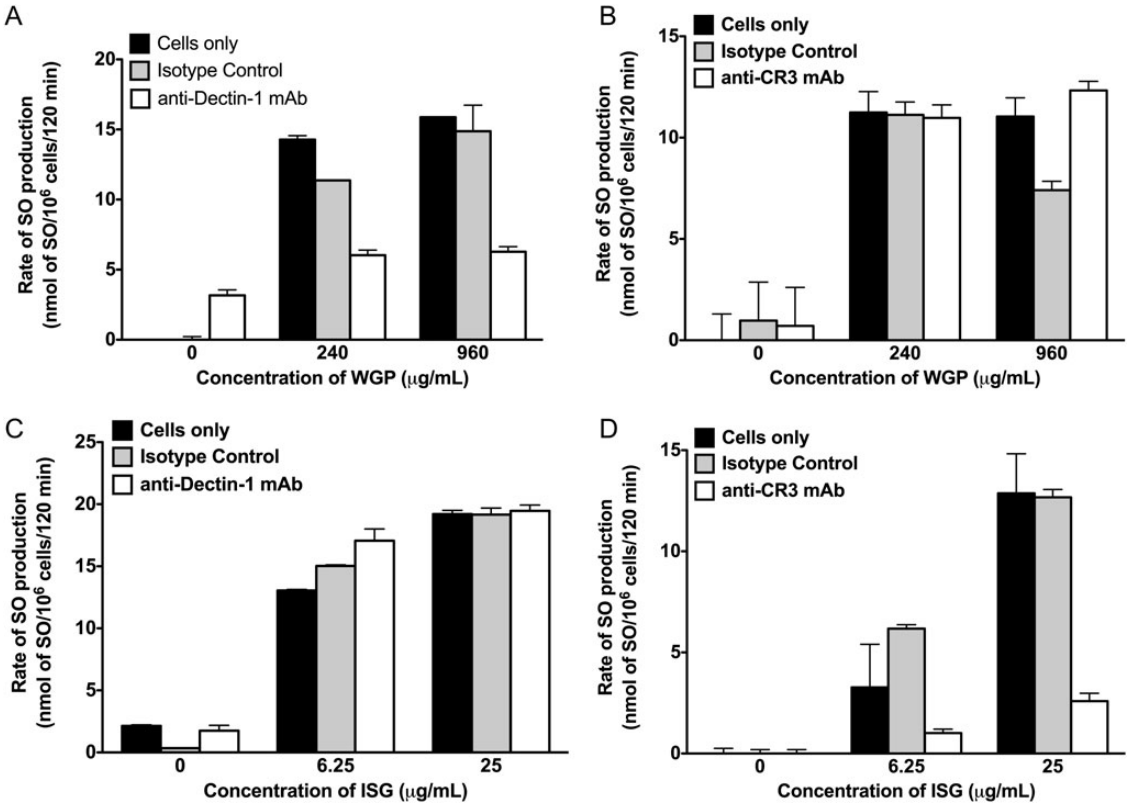

Fig. 4. The effect of receptor blocking on the WGP- and ISG-induced oxidative burst in human PBMC. The rate of SO production in human PBMC treated with Dectin-1 or CR3 blocking antibodies in response to stimulation with WGP (**A** and **B**, respectively) or ISG (**C** and **D**, respectively) was determined. Human PBMC were pretreated with either anti-Dectin-1 (GE2 at 10 μg/mL), anti-CR3 antibodies (LM2/1 at 10 μg/mL, VIM12 at 10 μg/mL and IB4 at 5 μg/mL) or equivalent isotype controls for 30 min at 4°C. Cells were subsequently stimulated with 0, 240 or 960 μg/mL of WGP or 0, 6.25 or 25 μg/mL ISG for 120 min at 37°C. The response of PBMC at each tested concentration of glucan is represented as rate of SO production (nmol of $SO/10^6$ cells/120 min). As a viability and specificity control, cells were stimulated with PMA. The oxidative burst activity induced by PMA was not inhibited by any of the blocking antibodies indicating that the inhibition effect was specific to the β-glucans (data not shown). Values plotted in the graph are mean values ± SD from triplicate wells in one experiment using one donor; these results are representative of at least two independent experiments from different donors.

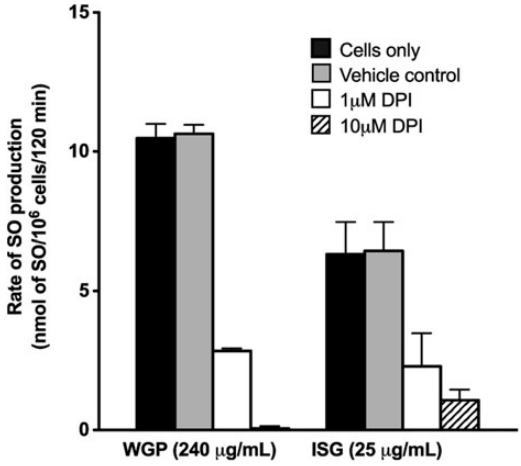

Fig. 5. Inhibition of WGP and ISG-induced oxidative burst by inhibitor of NADPH oxidase, DPI. Human PBMC pretreated with the NADPH oxidase inhibitor DPI (1 and 10 μM) at 4°C for 30 min, were stimulated with 240 μg/mL WGP or 25 μg/mL ISG at 37°C for 120 min and SO production was measured. The response of PBMC at each concentration of β-glucan is represented as rate of SO production (nmol of SO/10^6 cells/120 min). As the oxidative burst induced by PMA was also affected by DPI, a trypan blue exclusion test was performed to ensure that the cells were viable (data not shown). Values plotted in the graph are mean values ± SD from duplicate wells in one experiment using one donor; these results are representative of at least two independent experiments from different donors.

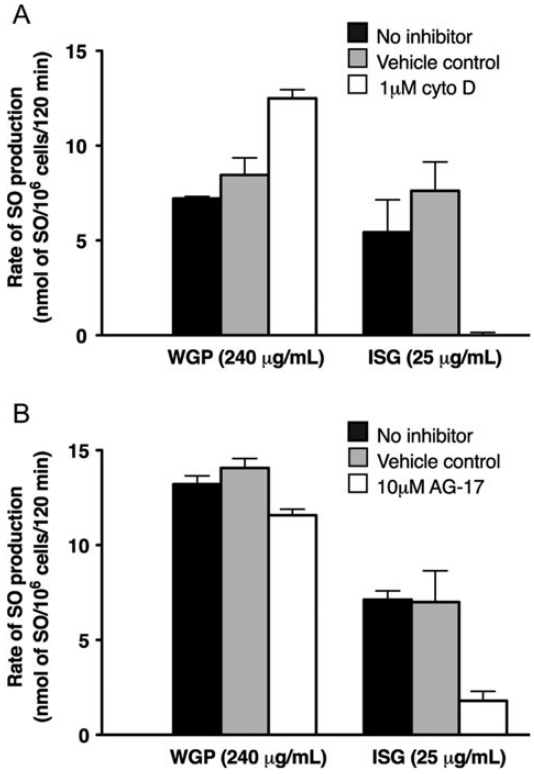

Fig. 6. The effect of cytochalasin D and FAK inhibitor on WGP- and ISG-induced oxidative burst. Human PBMC pretreated with (**A**) the actin polymerization inhibitor, cytochalasin D (1 μM), or (**B**) Pyk-2 focal adhesion kinase inhibitor, AG-17 (10 μM), or appropriate vehicle at 4°C for 30 min were stimulated with 240 μg/mL WGP or 25 μg/mL ISG at 37°C for 120 min and SO production was measured. The response of PBMC at each tested concentration of glucan is represented as rate of SO production (nmol of SO/10^6 cells/120 min). As viability control, cells were also stimulated with PMA. The oxidative burst induced by PMA was unaffected by the inhibitors at the concentration used (data not shown). Values plotted in the graph are mean values ± SD from duplicate wells in one experiment using one donor; these results are representative of at least two independent experiments from different donors.

isolated monocytes, we did all the subsequent signaling studies in PBMC. The signaling studies were not performed with isolated monocytes in order to avoid nonspecific activation of cells caused by manipulation of cells using magnetic beads (positive and negative selection).

While the interaction of pure β-1,3-oligosaccharide sequence with Dectin-1 has been investigated (Palma, et al. 2006), its interaction with CR3 is less known. Retention of the activity of ISG enriched for the 1,3-linked glucose units confirmed the specificity of the interaction with CR3 (Supplementary data, Figure S3).

Role of NADPH oxidase as the central mechanism of β-glucan-mediated oxidative burst in human PBMC

Next, we confirmed that the enzyme responsible for WGP- and ISG-induced oxidative burst in human PBMC was NADPH oxidase. The cells were assessed for the oxidative burst activity in response to these β-glucans in the presence of an NADPH oxidase specific inhibitor, diphenyleneiodonium (DPI). As shown in Figure 5, both the WGP- and ISG-induced oxidative burst rates were greatly reduced with 10 μM DPI, indicating that the oxidative burst induced by either form of β-glucan in human PBMC was NADPH oxidase-mediated.

Roles of actin polymerization and focal adhesion kinase in WGP- and ISG-induced oxidative burst in human PBMC

As Dectin-1 can function as a phagocytic receptor and CR3 is critical to phagocytosis and adhesion, we evaluated the importance of these cellular processes for the oxidative burst response. We therefore investigated the effect of inhibition of actin polymerization and focal adhesion kinase (FAK), the indirect measures of phagocytosis and adhesion, on WGP- and ISG-induced oxidative burst. Cytochalasin D, a toxin known to inhibit actin polymerization, was used to evaluate the role of actin remodeling; AG-17, a selective inhibitor of Pyk-2, the focal adhesion kinase protein present in monocytes, was used to assess the role of focal adhesion complexes in generation of oxidative burst (Bonvini et al. 1984; Kharazmi et al. 1989; Trial et al. 1995; Williams and Ridley 2000).

WGP-induced oxidative burst was not inhibited by 1 μM cytochalasin D (Figure 6A) and only slightly reduced by 10 μM of the Pyk-2 inhibitor, AG-17 (Figure 6B). In contrast, ISG-induced oxidative burst was completely abrogated by cytochalasin D (Figure 6A) and reduced by 75% with AG-17 (Figure 6B). AG-82, an inhibitor selective to FAK, the focal adhesion kinase considered to be absent in monocytes, did not affect the oxidative burst response (data not shown) (Li et al. 1998; Fuortes et al. 1999; Williams and Ridley 2000; Liu et al. 2004). These results collectively indicate that the WGP-induced respiratory burst in

Table II. List of pharmacologic inhibitors and their molecular targets

Inhibitor target	Compounds	Source
Actin polymerization	Cytochalasin D	Biomol (Philadelphia, PA)
Focal adhesion tyrosine kinases, PYK-2 and FAK	AG-17 and AG-82	Calbiochem (Gibbstown, NJ)
NADPH oxidase	DPI	Sigma-Aldrich (St. Louis, MO)
Src	PP2 and PP3	Calbiochem
Syk	BAY61-3606	Calbiochem
	Piceatannol	Sigma-Aldrich, Calbiochem
PI3K	LY 294002 and LY 303511	Calbiochem
Akt	Akt IV inhibitor	Calbiochem
p38 MAPK	SB 202190 and SB202474	Calbiochem
Protein kinase C	Staurosporine	Calbiochem

human PBMC does not require actin or focal adhesion kinase dependent processes, while they are necessary for ISG-induced activity.

Roles of SFK and Syk in WGP- and ISG-induced oxidative burst in human PBMC

We next evaluated the role of SFK and Syk in WGP- and ISG-induced oxidative burst in human PBMC. For inhibition of SFK, the cells were pretreated with PP2, the Src kinase selective inhibitor, or PP3, the inactive analog of PP2, as the negative control. BAY61-3606, the pharmacological inhibitor shown to inhibit Syk kinase was used to assess the role of Syk kinase in β-glucan-induced oxidative burst response (Yamamoto et al. 2003) (Table II).

The results illustrated in Figure 7A demonstrate that 10 μM PP2 completely inhibited both the WGP- and ISG-induced oxidative bursts, while the inactive analog, PP3, at the same concentration, did not affect the response, indicating that SFK plays a significant role in the respiratory burst response induced by both the β-glucans. In contrast, BAY 61-3606 at concentrations of 1 and 10 μM (concentrations more than 10-fold higher than the IC_{50} [half maximal inhibitory concentration] of 10 nM) did not inhibit WGP-induced oxidative burst activity, while ISG-induced activity was inhibited by ~80% with a concentration as low as 5 nM (Figure 7B). These results indicate that Syk is important for only the ISG-induced burst. It is important to note that Piceatannol (from two vendors), the commonly used Syk inhibitor in the literature, could not be used in these studies as it induced oxidative burst by itself when added to the cells.

Roles of PI3K, Akt and p38 MAPK in WGP- and ISG-induced oxidative burst in human PBMC

To investigate the role of the PI3K/Akt signaling pathway, PBMC were assessed for oxidative burst after treatment with 5 and 50 μM LY294002, the PI3K inhibitor, or after treatment with 50 μM LY303511, the inactive analog of LY294002. 5 and 10 μM Akt inhibitor IV, which selectively inhibits a kinase upstream of Akt and downstream of PI3K, was used to evaluate the involvement of Akt. The role of p38 MAPK was evaluated by using 1 or 10 μM of the inhibitor SB202190 and comparing the results with that of 10 μM SB 202474, the inactive analog of SB202190. The results presented in Figure 8A and B show that ~70% of WGP-induced oxidative burst was inhibited by 50 μM LY294002, while ~85% inhibition was achieved by the inhibitor Akt IV at 10 μM. Both the PI3K and Akt inhibitors were able to completely abrogate the ISG-induced oxidative burst. In contrast, the p38 MAPK selective inhibitor, SB202190,

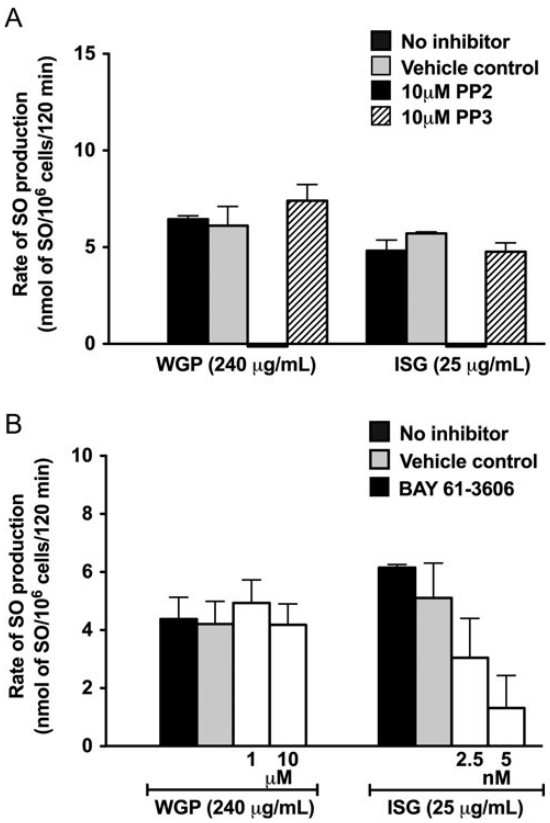

Fig. 7. The effect of inhibitors of SFK and Syk kinase on WGP- and ISG-induced oxidative burst. Human PBMC pretreated with (**A**) SFK inhibitor, PP2 (10 μM), PP3, inactive analog of PP2 (10 μM), (**B**) Syk kinase inhibitor, BAY61-3606 (1, and 10 μM for WGP experiments, and 2.5 and 5 nM for ISG experiments) or appropriate vehicle at 4°C for 30 min were stimulated with 240 μg/mL WGP or 25 μg/mL ISG at 37°C for 120 min and SO production was measured. The response of PBMC at each tested concentration of β-glucan is represented as rate of SO production (nmol of SO/10^6 cells/120 min). As viability control, cells were stimulated with PMA. The oxidative burst induced by PMA was unaffected by the inhibitors at the concentration used (data not shown). Values plotted in the graph are mean values ± SD from duplicate wells in one experiment using one donor; these results are representative of at least two independent experiments from different donors.

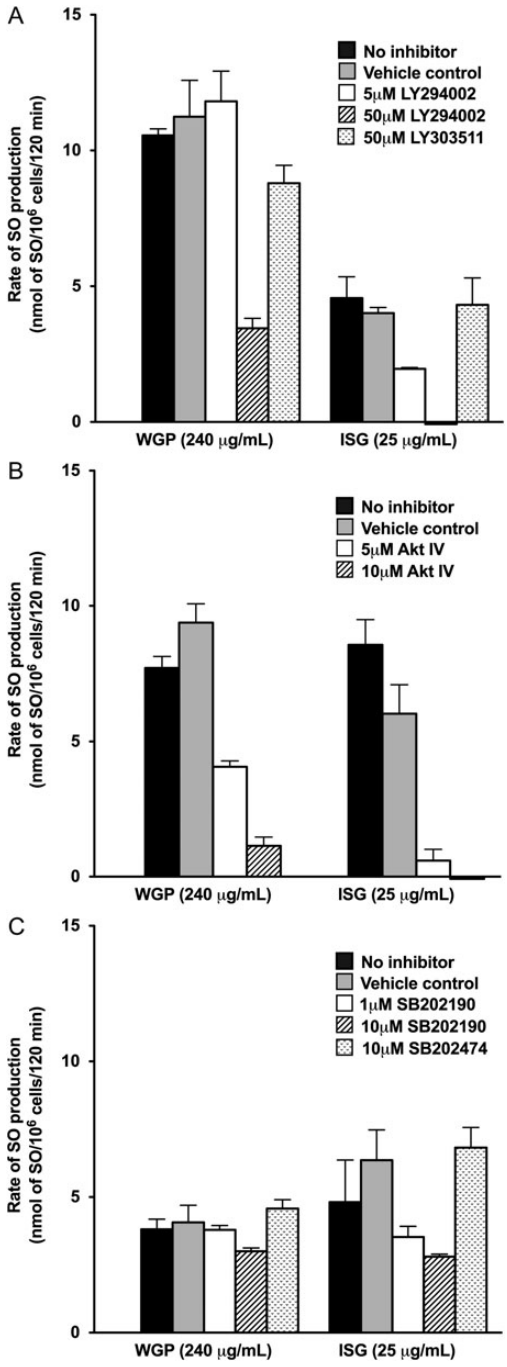

Fig. 8. The effect of inhibitors of PI3K, Akt and p38 MAPK in WGP- and ISG-induced oxidative burst. Human PBMC pretreated with (**A**) PI3K inhibitor, LY294002 (5 and 50 μM), or LY303511, the inactive analog of LY294002 (50 μM), or (**B**) Akt inhibitor, Akt IV (5, and 10 μM) or (**C**) p38 MAPK inhibitor, SB202190 (1 and 10 μM), or the inactive analog of SB202190, SB202474 (10 μM), or appropriate vehicle at 4°C for 30 min were stimulated with 240 μg/mL WGP or 25 μg/mL ISG at 37°C for 120 min and SO production was measured. The response of PBMC at each tested concentration of glucan is represented as rate of SO production (nmol of SO/10^6 cells/120 min). As viability control, cells were also stimulated with PMA. The oxidative burst induced by PMA was unaffected by the inhibitors at the concentration used (data not shown). Values plotted in the graph are mean values ± SD from duplicate wells in one experiment using one donor; these results are representative of at least two independent experiments from different donors.

at the concentrations tested resulted in no inhibition of the WGP-induced oxidative burst, while caused an ~40% reduction of ISG-induced activity (Figure 8C). These findings show that PI3K/Akt signaling molecules are essential for both WGP- and ISG-induced oxidative burst, while p38 MAPK plays a role in the induction of oxidative burst only by ISG.

Role of the phosphatidylinositol signal transduction system, including PLCγ and protein kinase C in WGP- and ISG-induced oxidative burst in human PBMC

The importance of the phospholipase C-protein kinase C (PLC-PKC) signaling axis was investigated using U73122 and staurosporine, common inhibitors to PLCγ and PKC, respectively. Figure 9A and B shows that neither U73122 (at up to 1 μM) nor staurosporine (10 nM) inhibited WGP-induced oxidative burst activity. In contrast, PLC-PKC signaling proved to be essential for ISG-induced oxidative burst, as the activity was inhibited by these two compounds (Figure 9).

Discussion

Two different forms of β-glucan, particulate and immobilized soluble β-glucan, have been shown to induce the oxidative burst response in mouse and human phagocytic cells (Michalek et al. 1998; Lavigne et al. 2006). These studies, however, did not interrogate the receptors or signaling pathways utilized by these two different forms of β-glucans. Since CR3 and Dectin-1 are heavily implicated as β-glucan receptors, the roles of these two receptors were evaluated in this study using purified and characterized β-glucans WGP and ISG as the ligands and oxidative burst as the functional cellular readout. Note that, there is no consensus on the ability of CR3 or Dectin-1 to recognize a particular form of β-glucan. Both Dectin-1 and CR3 have been reported to bind particulate and immobilized β-glucans (Cain et al. 1987; Ross et al. 1987; Thornton et al. 1996; Xia and Ross 1999; Willment et al. 2001; Brown et al. 2002; Willment et al. 2005; Lavigne et al. 2006; Palma et al. 2006; Adams et al. 2008; Ujita et al. 2009; van Bruggen et al. 2009). Possible explanations for the lack of consensus in conclusively determining the receptor for the various β-glucan forms include: (a) purity of β-glucan preparations, (b) variations in β-glucan structure, (c) differences between Dectin-1 and CR3 in humans and mice and (d) differences between cell types such as monocytes and neutrophils. Regardless, our results demonstrated that in human mononuclear cells Dectin-1 was important for oxidative burst induced by WGP, whereas CR3 was critical to oxidative burst induced by ISG. Despite the use of different receptors, both β-glucan forms utilized a final common NADPH oxidase pathway.

Regarding oxidative burst induced by phagocytizable WGP, our findings are not consistent with others, which demonstrated zymosan or intact yeast can activate human neutrophils via CR3 (Cain et al. 1987; van Bruggen et al. 2009). Besides the obvious differences in cell type and purity of stimulus, it is possible that (a) anti-Dectin-1 antibody is a more effective blocking antibody than the CR3-specific antibodies and (b) laminarin, used as Dectin-1 blocking agent in earlier studies, is not very effective as laminarin has been shown to bind both CR3 and Dectin-1 (Ross et al. 1985; Xia and Ross 1999).

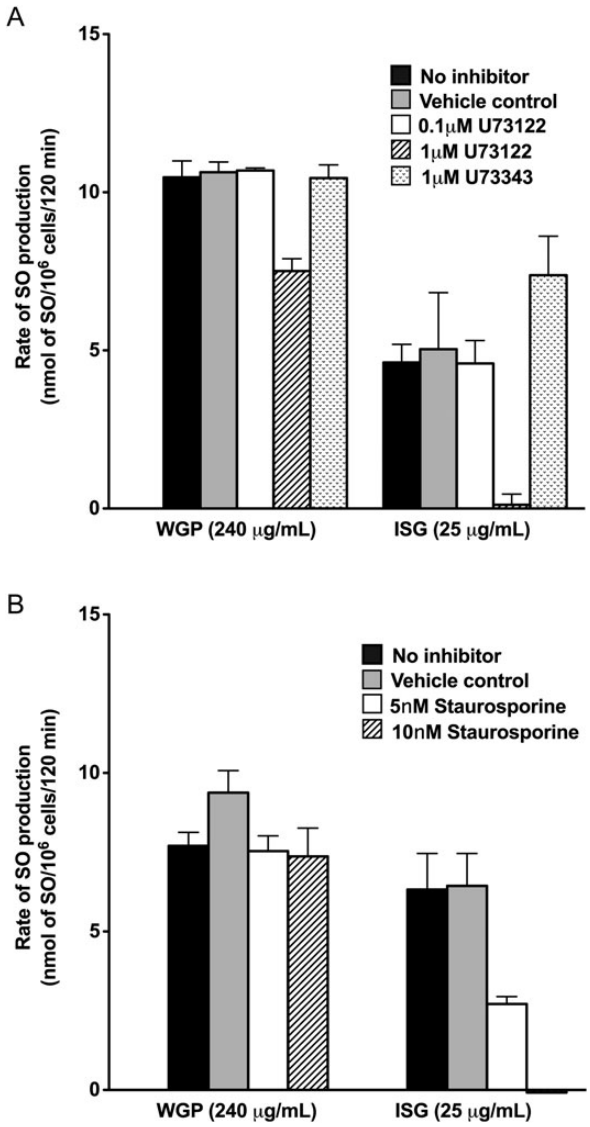

Fig. 9. The effect of inhibitors of PLCγ and PKC in WGP- and ISG-induced oxidative burst. Human PBMC pretreated with (**A**) PLCγ inhibitor, U73122 (0.1 and 1 μM) or U73343, inactive analog of U73122 (1 μM), or (**B**) PKC inhibitor, Staurosporine (5 and 10 nM), or appropriate vehicle at 4°C for 30 min were stimulated with 240 μg/mL WGP or 25 μg/mL ISG at 37°C for 120 min and SO production was measured. The response of PBMC at each tested concentration of glucan is represented as rate of SO production (nmol of SO/10^6 cells/120 min). As viability control, cells were also stimulated with PMA. The oxidative burst induced by PMA was unaffected by the PLCγ inhibitors at the concentration used (data not shown). In inhibition studies using staurosporine, the PMA control was affected; therefore, the viability of the cells was checked by the trypan blue method (data not shown). Values plotted in the graph are mean values ± SD from duplicate wells in one experiment using one donor; these results are representative of at least two independent experiments from different donors.

Regarding plate-bound β-glucan, Dectin-1 has been shown to have a role in oxidative burst response only in murine dendritic cells (Goodridge et al. 2011). Our results demonstrate that human monocytes utilize CR3 to produce oxidative burst in response to plate-bound β-glucan, and interestingly, ISG-induced oxidative burst was complement-independent. The differences in the results of this study compared with those shown in the literature may be due to differences in species or cell types.

Since other putative β-glucan receptors have been identified, such as lactosylceramide, CD5 and scavenger receptors, further investigation on these receptors needs to be done using highly purified, well-characterized β-glucans in order to delineate their significance in β-glucan recognition.

The role of CR3 and Dectin-1 in induction of oxidative burst in response to different forms of β-glucan was further confirmed by evaluating the molecules that have been previously shown to be associated with downstream signaling from the respective receptors. In terms of critical cellular processes including phagocytosis and adhesion associated with Dectin-1 and CR3, inhibition of actin polymerization by cytochalasin D did not affect WGP-induced oxidative burst response; instead, a trend toward increased oxidative burst response was seen (Figure 6A). This observation was consistent with earlier published results where after cytochalasin D treatment a Dectin-1 overexpressing cell line still bound zymosan without internalization and produced enhanced levels of cytokine (Brown et al. 2003). This increase in response could be attributed to cytochalasin D-induced inhibition of Dectin-1 internalization leading to sustained signaling (Hernanz-Falcón et al. 2009). Consistent with an integrin-mediated event, ISG-induced oxidative burst was completely inhibited by blocking either actin polymerization or focal adhesion kinase (Figure 6), suggesting a possible role for cell adhesion to the immobilized β-glucan. It is also possible that in human PBMC CR3 is merely facilitating adhesion of cells to the plate allowing β-glucan to interact with other low-affinity β-glucan receptors.

The results of the signaling inhibitor studies described here suggest that WGP (Dectin-1)- and ISG (CR3)-induced oxidative burst are the consequence of distinct but overlapping signaling pathways (Figures 7–9). The activity of these signaling pathways in Dectin-1 and CR3 signaling after β-glucan ligation has been observed in other model systems albeit with some differences. While Syk involvement in Dectin-1 mediated signaling was not observed in our study (Figure 7B), it has been demonstrated for signaling in primed murine macrophages for ROS production (Underhill et al. 2005) as well as for phagocytosis in murine fibroblast but not a macrophage-like cell line (Herre et al. 2004).

The involvement of the PI3K/Akt signaling pathway in both Dectin-1 and CR3-mediated oxidative burst induction (Figure 8A and B) is generally consistent with previously published studies regardless of species differences (Li et al. 2006; Shah et al. 2009).

Some differences from our results describing the role of p38MAPK in signaling downstream of Dectin-1 have been reported, where p38MAPK was shown by others to be important in Dectin-1 signaling in murine bone marrow-derived macrophages although not in dendritic cells (Goodridge, Shimada et al. 2009; Xu, Huo et al. 2009). It should be noted that in these studies zymosan was used as the β-glucan stimulus, and zymosan used in these studies differs greatly in β-glucan purity from the WGP used in this study.

Our study demonstrates that the PLCγ-PKC pathway is important for CR3-mediated oxidative burst induction and suggests that the same pathway has little importance for Dectin-1-mediated

oxidative burst induction (Figure 9). However, Xu, Gunawan et al. (2009) has been reported that cytokine production by zymosan stimulation of Dectin-1 on murine bone marrow-derived dendritic cells does signal via the PLCγ-PKC pathway. These discrepancies could be because the signaling molecules involved in Dectin-1-mediated functions depend on the nature of the ligand, the cell type, the species involved and the nature of the response.

In the context of immune recognition and response to PAMPs, human mononuclear cell oxidative burst induced by ISG required lower β-glucan concentrations than oxidative burst induced by WGP. Similar results were shown using rat macrophages and human neutrophils (Michalek et al. 1998; Lavigne et al. 2006). The difference in the threshold of immune activation by WGP and ISG may have biological significance, because a parallel can be drawn between WGP or ISG and the phagocytizable yeast form or the nonphagocytizable filamentous form of pathogenic dimorphic fungi, respectively.

Human phagocytes have been shown to differentially respond to the phagocytizable conidial forms versus nonphagocytizable filamentous yeast forms of a pathogenic dimorphic fungus. Some of the examples are: The filamentous form of *Candida albicans* has been shown to be more virulent compared with its yeast counterpart and the heightened sensitivity of immune cells to the hyphal form of fungus is critical to its elimination (Calderone and Fonzi 2001; Lavigne et al. 2006). Priming of macrophages by interferon gamma has been shown to have different killing activity against the phagocytizable and nonphagocytizable form of *Candida albicans* (Brummer et al. 1985). In case of *Blastomyces dermatidis*, human phagocytes are more efficient in ingesting and killing the conidial form before it develops into the yeast form and causing blastomycosis (Deppe 1989). More recently, the role of β-glucan in activation of IL-23/TH-17 axis by the hyphal form of opportunistic fungi in human versus its pathogenic form was described (Chamilos et al. 2010).

A greater understanding of the β-glucan recognition and downstream signaling mechanisms in human PBMC increases our ability to evaluate the host defense mechanisms evoked by the different forms of pathogenic and opportunistic fungal infections and thereby help design better therapeutic fungal targets.

In summary, the present study provides insight into the molecular mechanisms involved in β-glucan-induced oxidative burst in human PBMC (Figure 10). We show that different physical forms of β-glucan stimulate distinct receptors, CR3 or Dectin-1. We also demonstrate that these receptors use different, but overlapping, signaling pathways to ultimately induce an oxidative burst response by a common NADPH oxidase-dependent reaction. This study demonstrates the importance of the manner of presentation of β-glucan to glucan receptor-bearing immune cells for eliciting a functional response and highlights the caveats of using the results of a study with one form of β-glucan to predict the outcomes of other studies using different physical forms (or sources) of β-glucan.

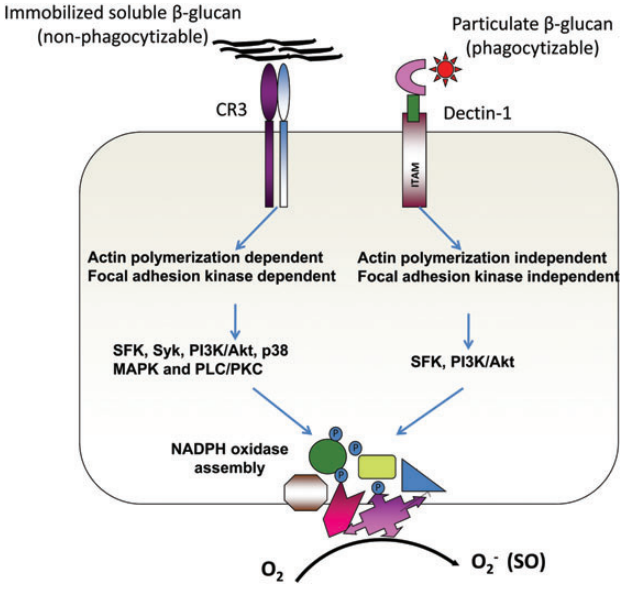

Fig. 10. Hypothetical model showing the two distinct but overlapping pathways induced by different structural forms of β-glucan in the production of SO.

Materials and methods

β-Glucans used and their characterization

Saccharomyces cerevisiae-derived WGPs and soluble β-glucan (average MW ~150 kDa) were prepared and characterized by Biothera (Eagan, MN). Preparation of particulate β-glucan has been described in earlier papers (Li et al. 2007; Goodridge et al. 2011). Soluble β-glucan was prepared by acid hydrolysis of WGP and fractionated by preparative gel permeation chromatography (GPC). The β-glucans used were characterized analytically with respect to the parameters listed in Table I. The β-glucans were determined to be endotoxin-free and then used in the assay system on the basis of hexose concentration. Soluble yeast mannan (Sigma-Aldrich, St. Louis, MO) was prepared by dissolving in PBS.

Preparation of soluble β-glucan enriched in β-1,3-glucose-linked units was done by treating 50 mg of soluble glucan at 5 mg/mL in a sodium acetate pH = 5 buffer with 9.6 units of 1,6-glucanase (pustulanase from Prokazyme, Iceland) at 80°C for 25 min. The resulting solution was dialyzed over a 3K Amicon centrifugal filter to remove the digested 1,6-linked material and to afford the 1,3-enriched material. This treatment reduced the 1,6-linked glucose residues from 2.6 to 0.4%. As control, an equal amount of the enzyme added to dextran solution, an inactive carbohydrate source was also tested. The lack of activity of this enzyme control ensured that the response from the soluble β-glucan enriched in β-1,3-glucose-linked units is not due to the enzyme contaminated with β-1,3-glucose-linked β-glucan (data not shown).

Study population and PBMC sources

The study was approved by the New England Institutional Review Board (Wellesley, MA). Blood was collected from healthy male and female volunteers who had signed an Informed Consent Form (ICF) prior to blood collection.

PBMC isolation

PBMC were isolated by density gradient centrifugation using Histopaque 1077 (Sigma-Aldrich). Heparinized whole blood (10 U/mL) was diluted 1:2 with 1 × Dulbecco's phosphate-

buffered saline (DPBS) without calcium and magnesium (Mediatech, Inc., Herndon, VA). Aliquots of ~20 mL of diluted cell preparations were transferred to sterile 50 mL conical tubes. The diluted cell preparation was subsequently underlayed with 12 mL of Histopaque 1077 and the tubes were centrifuged without applying brakes at 2400 rpm (~800 × *g*) for 20 min at room temperature. The PBMC were removed using a sterile transfer pipette and transferred to a sterile 50 mL conical tube. The cells were washed twice by bringing the volume up to 50 mL with 1 × DPBS and centrifuging at 1700 rpm (~500 × *g*) for 5 min. The cell pellet was resuspended in 10–20 mL of 1 × DPBS counted with a hemacytometer. In some of the experiments, the PBMC preparation was further purified by lysing the red blood cells by osmotic shock.

Monocyte depletion and enrichment

Monocytes were depleted from PBMC using CD14 microbeads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. Briefly, PBMC were washed and resuspended at 10^7 cells per 80 µL in cell selection buffer (PBS supplemented with 0.5% bovine serum albumin (BSA) and 2 mM ethylenediaminetetraacetic acid (EDTA)). CD14 microbeads was added to PBMC at 20 µL per 10^7 PBMC, mixed and incubated at 2–8°C for 15 min. After incubation, the cells were washed once in the selection buffer, resuspended at up to 10^8 cells in 500 µL of buffer and subsequently allowed to pass through a magnetic separation LS column (Miltenyi Biotec). Eluate containing the monocyte-depleted fraction was collected and assessed for the extent of depletion by flow cytometry using a CD14-specific Ab.

For monocyte enrichment, Dynabeads® Untouched™ Human Monocytes (Invitrogen, Oslo, Norway) was used according to the manufacturer's instructions. In short, PBMC were washed and resuspended at 10^8 cells per mL in cell selection buffer. Blocking reagent and antibody mix were subsequently added at a concentration of 100 µL per 5×10^7 cells and incubated for 20 min at 2–8°C. The cells were washed once and incubated with 500 µL washed Depletion MyOne SA Dynabeads for 15 min at 2–8°C. After incubation, the cells were resuspended thoroughly and mixed with an additional 5 mL of selection buffer. The tube containing the cells was placed in the magnet for 2 min, the supernatant containing the untouched CD14+ cells was collected and assessed for purity using a CD14-specific Ab.

Immunofluorescent staining of cells

Freshly purified PBMC were resuspended in 0.1 mL immunofluorescence buffer (Hank's balanced salt solution containing 1% fetal bovine serum and 0.1% sodium azide) with 5–10 µL of Fc receptor (FcR) block (Miltenyi Biotec) and incubated at 4°C for 10 min. After the FcR block step, staining antibodies to CD14, CR3 or Dectin-1 were added to the cells and incubated at 4°C for 30 min. Monocytes were stained with CD14-Pacific Orange (BioLegend, San Diego, CA). Dectin-1 was detected on monocytes and neutrophils by staining with GE2 (AbD Serotec, Raleigh, NC) as the primary antibody, followed by PE-labeled goat anti-mouse at 1 µL as the secondary antibody (BioLegend). CR3 was stained with PE/Cy5 labeled ICRF44 (BioLegend). After incubation, the cells were washed twice with immunofluorescence buffer and analyzed by flow cytometry.

Treatment of cells with receptor-blocking antibodies

For blocking the CR3 receptor, the cells were preincubated with specific receptor-blocking antibodies or the relevant isotype controls at 4°C for 30–45 min before exposure to WGP or ISG and measurement of oxidative burst. For CR3 blocking, combination of antibodies that were used were (i) LM2/1, a mouse anti-human IgG1 monoclonal antibody to the I domain of CD11b chain of CR3 (10 µg/mL; Bender MedSystems, Burlingame, CA), (ii) VIM12, a mouse monoclonal IgG1 anti-human antibody to the lectin domain of CD11b chain of CR3 (10 µg/mL; Caltag Laboratories, Burlingame, CA), and (iii) IB4, a mouse monoclonal IgG2a anti-human antibody to the CD18 chain of CR3 (10 µg/mL; Ancell, Bayport, MN). The blocking antibody concentrations were determined using dose titration (log dilutions) in the oxidative burst assay. The lowest antibody concentration where the blocking activity plateaued was chosen as the concentration used in all subsequent blocking experiments. All the isotype controls, mouse IgG1 (clone P3; eBioscience, San Diego, CA) and mouse IgG2a (clone eBM2A; eBioscience) were used at the same concentration as the blocking antibodies. Dectin-1 blocking antibody GE2 was provided by Dr. Gordon Brown, University of Aberdeen, UK (Willment et al. 2005).

Treatment of cells with pharmacological inhibitors of signaling molecules

To inhibit the signaling molecules, the cells were incubated with the pharmacological inhibitors listed in Table II at the indicated concentrations, or the appropriate vehicle (dimethyl sulfoxide in all cases), at 4°C for 30–45 min before exposure to WGP or ISG and the measurement of oxidative burst.

Oxidative burst assay

WGP (15–1920 µg/mL) and soluble β-glucan (0.7–200 µg/mL) were prepared at 2-fold out-of-plate serial dilutions in DPBS. Subsequently, 100 µL of each dilution were added to triplicate wells of Costar® Universal-Bind™ microtiter plates (Corning, Lowell, MA). For immobilizing the soluble β-glucan on the plate, the plate was incubated at 37°C in a humidified incubator with 5% CO_2 for 60 min to allow passive adsorption. After incubation, the plates with the soluble β-glucan were emptied by flicking and irradiated for 5 min in a UV transilluminator (120,000 µJ/cm^2) to facilitate the covalent binding between the plate and the β-glucan. The microtiter plates were then blocked with 0.25% BSA in DPBS for 30 min at room temperature. Each microtiter plate included positive control wells and negative control wells to which only DPBS with no β-glucan was added.

The oxidative burst response was determined by standard methods (Mayo and Curnutte, 1990) by measuring SO production through the reduction of cytochrome c. PBMC were resuspended in Hanks' balanced salt solution (HBSS)/*N*-(2-hydroxyethyl) piperazine-*N*′-(2-ethanesulfonic acid) (HEPES) buffer with 0.25% BSA at a concentration of 4×10^6 cells/mL and maintained at 37°C until added to the plate. A 100 µL aliquot of 200 µM bovine cytochrome c (Sigma-Aldrich) solution in HBSS/HEPES buffer previously incubated at 37°C was added to each well. Approximately 4.0×10^5 cells, in a 100 µL volume, were subsequently added to each well of the microtiter plate. The negative assay control, consisting of cells and cytochrome c, was added in

the uncoated negative control wells. As a positive assay control, just before reading the plate, phorbol myristate acetate (PMA) at 100 ng/mL was added as the stimulus to the cells in the uncoated positive control wells to achieve a final concentration of 50 ng/mL. Serum was not used in any of the assay buffers as presence of complement proteins could lead to potential misinterpretation of the data. The plate was maintained at 37°C, and optical density (OD) in each well was read at 550 nm every 15 min for 120 min using a spectrophotometer (Spectramax 250, Molecular Devices, Sunnyvale, CA).

For all studies using pharmacological inhibitors PMA stimulation served as a viability control, ensuring that any observed inhibition of oxidative burst was not due to cell death. Inhibitor concentrations that did not affect the cellular response to PMA were considered nontoxic. In cases where the inhibitors affected the cellular response to PMA because the signaling molecules played a role in PMA-induced response (e.g. PKC), the viability of the cells was assessed by trypan blue exclusion.

Data analysis and representation

For each concentration of β-glucan, the OD change (Δ OD) was calculated from the time of minimum response (1 min) to the time of maximum response (120 min). The rate of SO production (nmol of SO/10^6 cells/120 min) was calculated from the Δ OD value and the extinction coefficient of cytochrome c (21.1×10^3/M/cm). The results presented in all figures are representative of at least two independent experiments from two different donors.

Supplementary data

Supplementary data are available online at *http://glycob.oxfordjournals.org/*.

Authors' contributions

L.R.W., M.L.L. and C.M.D. performed all the oxidative burst assays. M.E.D. and P.M.W. performed the analytical characterization of the β-glucans. A.S.C. carried out the purification of monocytes for some of the experiments. N.B. and J.P.V. participated in the design and coordination of the study. N.B., J.P.V., A.S.C., S.E.N., M.L.P., J.J.D.L. and F.J.L. drafted the manuscript. All the authors read and approved the final manuscript.

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Conflict of interest

N.B., L.R.W., A.S.H.C., M.E.D., P.M.W., S.E.N. and M.L.P., are employees of Biothera and are beneficiaries of the Biothera employee stock plan. C.M.D. and M.L.L. are former employees of Biothera. J.P.V. is a former employee of Biothera and currently receives consulting fees from Biothera. J.J.D.L. and F.J.L. have no competing interests. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed. Opinions, interpretations, conclusions and recommendations are those of the authors and are not necessarily endorsed by the US Army. The mention of trade names, commercial products or organizations does not imply endorsement by the US Government.

Abbreviations

BSA, bovine serum albumin; CR3, complement receptor type 3; DPBS, Dulbecco's phosphate-buffered saline; DPI, diphenyleneiodonium; EDTA, ethylenediaminetetraacetic acid; FAK, focal adhesion kinase; GPC, gel permeation chromatography; HBSS, Hanks' balanced salt solution; HEPES, *N*-(2-hydroxyethyl) piperazine-*N*′-(2-ethanesulfonic acid); ICF, Informed Consent Form; ISG, immobilized soluble β-glucan; MAPK, mitogen activated protein kinase; NADPH, nicotinamide adenine dinucleotide phosphate; OD, optical density; PBMC, peripheral blood mononuclear cells; PI3K, phosphatidylinositol-3 kinase; PKC, protein kinase C; PLC, phospholipase C; PMA, phorbol myristate acetate; ROS, reactive oxygen species; SFK, Src family kinase; SO, superoxide; Syk, spleen tyrosine kinase; WGP, whole glucan particle.

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